ANTIMICROBIAL SUSCEPTIBILITY AND POPULATION DYNAMICS OF A DEFINED BIOFILM COMMUNITY UNDER DIFFERENT NUTRIENT CONDITIONS

KERSTIN GARNY

Thesis presented in partial fulfillment of the requirements for the degree of Master of Science at the University of Stellenbosch

Supervisor G.M. Wolfaardt

April 2004

Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Little is known about the impact of nutrient conditions on antimicrobial resistance in biofilms grown under continuous flow conditions. Furthermore, community-level response of biofilms to antimicrobial substances and different nutrient regimes are poorly described. A better understanding of the influence of environmental conditions on biofilm behavior and antimicrobial susceptibility may contribute to the efforts, addressing the problems associated with increased antimicrobial resistance. Therefore, the aim of this study was to evaluate the survival and population dynamics in a defined mixed-species biofilm community grown under different nutrient conditions and when subjected to biocide treatment.

Epi-fluorescence microscopy in conjunction with the LIVE/DEAD® *Bac*Light™ viability kit, a conventional cultivation technique (plate counts), and culture-independent techniques (terminal restriction fragment length polymorphism and fluorescent *in situ* hybridization) were applied to observe biofilm and planktonic antimicrobial susceptibility, as well as population dynamics. A defined mixed-species community, consisting of four bacterial strains, was cultivated and monitored in a flow cell system. Two nutrient types were used: 1) a complex growth medium [tryptone soy broth (TSB)] and 2) a defined synthetic medium [minimal salts supplemented with glucose (MSM + Glucose)]. In addition, these two nutrient types were applied in different concentrations.

Biofilm and planktonic community behavior was influenced by the nutrient type and concentration. Species evenness in the planktonic community was influenced by the nutrient conditions, while species richness changed in response to biocide treatment and nutrient conditions. TSB-grown microbial communities were more susceptible directly after biocide treatment than those grown in MSM + Glucose, however, biofilm viability in the latter nutrient condition decreased within 24 h after biocide treatment. Furthermore, a surprising difference in the recovery rate between biofilm and associated planktonic communities was observed. A conceptual model was developed that aimed to explain the observed biofilm-planktonic interactions. This model proposes that the cells found in the outer regions of a biofilm are the primary source of the associated planktonic cells, and that this phenomenon is independent from overall biofilm activity.

OPSOMMING

Daar is tans min bekend oor die invloed van nutriënte op die antimikrobiese weerstandbiedendheid van biofilms onder kontinue vloeitoestande. Verder is daar 'n groot leemte in die literatuur oor die invloed van nutriënte op die antimikrobiese sensitiwiteit van bakteriese gemeenskappe in multispesie-biofilms. 'n Beter begrip van die invloed van omgewingsfaktore op biofilmgedrag en antimikrobiese sensitiwiteit sal bydra om die probleem van verhoogde antimikrobiese weerstandbiedendheid te beheer. Die doel van hierdie studie was gevolglik om die oorlewingsvermoë, asook die gemeenskapverskuiwings binne 'n bekende biofilmgemeenskap te evalueer, nadat dit blootgestel was aan biosiedbehandeling en verskillende nutriëntkondisies.

Epifluoressensie-mikroskopie in kombinasie met 'n lewensvatbaarheidskleurstof (LIVE/DEAD® *Bac*Light™ viability kit), konventionele kweektegnieke (spreiplaattegniek) en kweek-onafhanklike identifikasietegnieke (terminale restriksie fragment lengte polimorfisme en fluoressensie *in situ* hibridisasie) is gebruik om biofilm- en planktoniese lewensvatbaarheid, asook gemeenskapverskuiwings te bestudeer. 'n Gedefinieerde biofilmgemeenskap, bestaande uit vier verskillende bakteriese rasse, is gekweek en gemonitor in 'n vloeiselsisteem. Twee tipes nutriënte is gebruik: (1) 'n komplekse groeimedium [Tryptone Soy Broth (TSB)] en (2) 'n chemies-gedefinieerde medium [minimale soutoplossing met glukose (MSM + Glucose)]. Beide nutriënttipes is in verskillende konsentrasies toegedien.

Die gedrag van biofilm- sowel as planktoniese populasies is beïnvloed deur beide die nutriënttipe en konsentrasie. Planktoniese populasiegetalle is beïnvloed deur nutriëntkondisies, terwyl die aantal populasies beïnvloed is deur biosiedbehandeling en nutriënttoestande. Bakteriese gemeenskappe wat in komplekse medium (TSB) gekweek is, was meer sensitief teenoor die biosied direk na behandeling as die wat in MSM + Glukose gekweek is. Die lewensvatbaarheid van die biofilm wat in MSM + Glukose gekweek is, het egter eers 24 uur na biosiedbehandeling begin daal. Daar was verder 'n opvallende verskil in die tempo van herstel van die biofilmgemeenskap in vergelyking met die planktoniese gemeenskap. Die planktoniese gemeenskap het vinniger herstel as die biofilm gemeenskap. 'n Model is ontwikkel om die biofilm-planktoniese interaksies te verklaar. In die model word voorgestel dat selle wat in die buitenste lae van 'n biofilm groei, die primêre bron is van planktoniese selle in 'n vloeisisteem, en dat hierdie verskynsel onafhanklik is van die algehele biofilm-aktiwiteit.

ACKNOWLEDGEMENTS

I thank my Lord and Savior, Jesus Christ, for an opportunity to learn and to grow, and for the blessing, these last couple of years have been.

Furthermore, I like to express my sincere gratitude to:

Prof. Gideon Wolfaardt for his guidance and support, as well as for his encouragements and believe in my abilities.

Dr. Lydia Joubert and Dr Murray Gardner, for their technical support and guidance.

My fellow colleagues in the Wolfaardt and Botha laboratories for their friendship, advice, encouragement and support, at all times.

Ms Ronel Augustyn, Sasol Technology, for her interest and support.

The staff and students of the Department of Microbiology at the University of Stellenbosch for their advice and support.

SASOL, National Research Foundation (NRF) and the Department of Microbiology of the University of Stellenbosch for their financial support.

My friends for their friendship

A special thanks goes to my family, especially my mother for her selfless support over my years of study, and my father for encouraging me to think for myself and to believe in myself.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

CHAPTER 1:
INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

There has been an increase in interest in antimicrobial resistance due to the importance of this phenomenon in industry and health care. Although it is widely accepted that biofilm populations are more resistant towards antimicrobials than suspended or planktonic microbial populations, there are claims that it is not always the case (Spoering and Lewis 2001). What are the reasons and mechanisms that cause biofilm microbial population survival, and their persistence once established in a system, e.g., the human body or a water distribution line? Many possible explanations have been proposed for the increased resistance among biofilms, but so far biofilm research has often supported the statement "The more we know, the more we know how little we know." Even single-species biofilms have proven to be complex biological systems, becoming more complex with increased microbial diversity. Indeed, the complexity of biofilms complicates the formulation of a defined action-reaction behavior (Allison et al 2000). Among the factors contributing to the complex nature of biofilms, are the microbial responses to nutrient status, heterogeneity in biofilm structure and associated EPS, as well as growth rate.

This complexity is seen in the cascading effect of one environmental factor such as nutrient availability. The nature, concentration and availability of nutrient and carbon sources have a profound influence on microbial behavior from biochemical reactions, to growth and reproduction, to survival mechanisms. Since biofilms are defined as attached cells in close proximity with each other, surrounded or embedded in an exopolymeric matrix, nutrient conditions not only influence the behavior of the individual cell, but also influences the overall biofilm composition and development, architectural and structural heterogeneity, microbial physicochemistry, and species diversity (Wolfaardt et al 1994a; Moller et al 1997). The chemical nature of available nutrients appears to have a significant impact on the growth potential and metabolic capability of a microbial community (Karthikeyan et al 1999). Nutrient availability is not only determined by nutrient concentrations or abundance, but also by the efficiency of nutrient transport (Xu et al 1998) within biofilms, as transport limitations

have been reported to promote starvation and slow growth due to nutrient or electron acceptor (oxygen) limitations (Xu et al 1998). It has been suggested that gradients in nutrient concentration result in corresponding gradients in growth rate or other physiological activities (Xu et al 2000). Karthikeyan et al (2001) studied the adaptation of bacterial communities to environmental transitions from labile to refractory substrates. The biofilm thickness and coverage decreased, and the architecture of the biofilms changed, when the substrates were shifted from labile to refractory. The same observations were made by Wolfaardt et al (1994a).

Biofilm structure and integrity is greatly influenced by the presence, type and amount of extracellular polymeric substances (EPS), which are also considered to determine the physicochemical and biological properties of the biofilm matrix (Wingender et al 1999). Wingender et al (1999) defined EPS as a general term for different classes of macromolecules such as polysaccharides, proteins, lipids, small amounts of nucleic acids and other polymers, which have been found in intercellular spaces between cells, cell aggregates or biofilm micro-colonies. Several studies showed that EPS production was partly influenced by nutrient sources and conditions (Christensen and Characklis 1990; Bonet et al 1993; Dewanti and Wong 1995; Looijesteijn et al 1999).

Despite evidence that growth rate is not the only factor responsible for antimicrobial susceptibility, it has been linked to antimicrobial resistance, with slower growth rates typically associated with increases in antimicrobial resistance (Brown et al 1988).

In the past, microbial ecological studies on microbial structures and diversity within the environment were limited by the unavailability of appropriate techniques, since most were dependent on the culturability of microorganisms. Accurate description of microbial community diversity and ecology requires the knowledge of species richness (number of species within the community), species evenness (size of bacterial population within a community) and the physiological role and interaction of each species with each other and its environment (Amann et al 1995). Culture-dependent techniques not only apply a selective pressure on environmental samples, but also change the natural conditions of the sample, thus only microorganisms able to adapt or grow in these artificial conditions are detected. The

inadequacy of culture-dependent techniques was demonstrated when culture-independent techniques, based on the genotypic differences of microbes, were applied (Amann et al 1995). Culture-independent techniques also confirmed the phenomenon of microbial cells being viable but non culturable, and opened the door for studying environmental microbial structure and diversity.

1.2 OBJECTIVES

Previous studies evaluated the influence of nutrient sources on growth rate, as well as development and structure of planktonic and biofilm microbial populations. Some of these studies related growth rate with antimicrobial susceptibility or antimicrobial resistance, although most studies involved single-species biofilms or biofilms cultivated on nutrient plates. Little research has been done combining these parameters within a system that allows observations at a surface-water interface. Therefore, the first aim of this study was to assess the influence of different nutrient conditions on the antimicrobial susceptibility of a defined mix-species biofilm and it's associated planktonic community within a flow cell system. The second aim was to determine the ability of biofilms to recover after biocide treatment under different nutrient conditions. The final aim was to study the influence of nutrient conditions and biocide treatment on the composition of a mixed-species biofilm.

The specific objectives were to:

1. Isolate several bacterial strains from cooling tower water by applying conventional culturing techniques to assemble a defined mixed-species community
2. Develop a protocol based on small scale flow cell microcosms to study the behavior of biofilm and planktonic phenotypes of this bacterial community
3. Combine culture-dependent and -independent techniques, epi-fluorescent microscopy and image analyses with flow cell studies to monitor the response of the community to biocide treatment under different nutrient conditions, in terms of viability, regrowth and population dynamics.

CHAPTER 2:
LITERATURE REVIEW

2.1 BIOFILMS

The term biofilm is used to describe the microbial form of sessile or surface attached cells, flocs ('planktonic biofilms') and sludge. Common to most types of biofilms is the presence of a matrix consisting of EPS in which microorganisms are imbedded (Flemming et al 2000). Traditionally, microorganisms have been considered to exist mostly as free-floating cells, but several decades ago it was proposed that microorganisms predominantly exist in association with surfaces. Since then, it has been discovered that biofilms are ubiquitous and are found on solid surfaces (substratum), at solid-water interfaces, solid-air interfaces and even at water-air interfaces (Flemming et al 2000).

2.1.1 Reason for biofilm formation

Microorganisms tend to establish in places with the highest nutrient concentrations. Organic and inorganic nutrients are often concentrated at surfaces of solid-water interfaces, which are therefore common places for biofilm growth, especially in nutrient poor environments. Other growth limiting factors could also stimulate biofilm growth, including unfavorable environmental conditions, such as high salt concentrations, temperature or toxic substances.

One of the greatest advantages for microorganisms living in association with each other in a biofilm is the production of EPS, which helps with initial attachment of surfaces and later the formation of a matrix in which the cells are embedded. Other functions of the EPS matrix include the creation of microenvironments, structural and architectural support, facilitating spatial cell arrangement, concentration of extracellular digestive and degradation enzymes, acting as a protective barrier against environmental changes or toxic compounds, or may serve as a trap for nutrients. EPS matrixes could be ideal environments for facilitating communication between cells, such as quorum sensing (Swift et al 1996), and the transfer of genetic material e.g., plasmids and transposons carrying antimicrobial resistance genes (Wingender et al 1999).

2.1.2 Biofilm development

The general model of biofilm development consists of several stages: 1) initial reversible attachment, followed by 2) irreversible attachment, leading to 3) biofilm maturation, which includes the increase of biomass and architectural changes (mushroom like structures) and then finally 4) detachment of cells from the biofilm (Watnick and Kolter 2000).

In recent years, more and more research has been undertaken in an attempt to understand the transition from planktonic to attached growth. The focus so far was primarily on the degree of gene regulation that is involved in the attachment mechanisms (Brözel et al 1995; Genevaux et al 1999; Prigent-Combaret et al 1999; Sauer and Camper 2001). From the results obtained it became clear that the transition from planktonic to sessile growth involves various and complex physiological changes (Sauer et al 2002).

Focusing on the work done by Sauer and coworkers (2002), it became evident that throughout the transitional process of biofilm development, from attachment, detachment and re-entry into planktonic growth, many physiological changes occur. Five stages of biofilm development in *Pseudomonas aeruginosa* were characterized with corresponding differences in gene expressions and protein regulation. The developmental stages were: 1) reversible attachment, 2) irreversible attachment, 3) maturation-1, 4) maturation-2, and 5) dispersion. A notable difference in phenotype between maturation-2 stage cells and the planktonic cells was observed, while the cells in the dispersion stage were more similar to the planktonic cell phenotype. The study indicated that *Pseudomonas aeruginosa* displayed multiple phenotypes with distinct physiological characteristics (metabolic and structural changes) that corresponded with the different stages of biofilm development. Biofilm cells were shown to change alginate production (EPS), regulation of motility and quorum sensing during the process of development. Prigent-Combaret et al (1999) also demonstrated that biofilm formation triggered differential expression of genes involved in EPS production, oxygen limitation, cell-to-cell signaling, osmolarity and motility. However, much work is still required to gain a better understanding and an improved characterization of the developmental process of biofilm formation.

2.1.3 Impact of biofilms

Due to their ubiquitous nature and their influence in industry and medicine, biofilms have become an important component of microbiological research. As often is the case in microbiology, the disadvantages associated with biofilms fueled the interest in biofilm formation, behavior and control. However, due to the numerous advantages of biofilms, such as their application in wastewater and sewage plants, bioremediation, and their beneficial role in our intestines, biofilms became a widely studied topic. Problems caused by biofilms include fouling of ship hulls, corrosion of water distribution systems, loss of heat exchange in water-cooling system, as well as attachment to medical devices and implants or causing diseases like cystic fibrosis (Morton et al 1998).

2.2 ANTIMICROBIAL SUSCEPTIBILITY OF BIOFILMS

Antiseptics, disinfectants and antibiotics have been applied for centuries to prevent or treat diseases and microbial growth. The resistance of microorganisms against antimicrobial treatments has become a great source of concern. Increasing numbers of microorganisms have shown antimicrobial resistance, especially in the medical field. The degree of the response towards antimicrobials varies between different types of microorganisms (McDonnell and Russell 1999). An extensive review was done by McDonnell and Russell (1999) on the action and activity of a variety of antimicrobials and the resistance of microorganism towards those compounds.

2.2.1 Antimicrobial resistance of attached vs planktonic microorganisms

Not only does the degree of antimicrobial resistance vary due to different types of microorganisms, but also as early as in 1984, LeChevallier et al (1984), and Nickel and coworkers (1985) observed that microorganisms growing in biofilms showed decreased sensitivity towards biocides and antibiotics. Since then, this phenomenon of reduced antimicrobial susceptibility of biofilms has received much attention (Costerton et al 1987; Exner et al 1987; Nichols 1989). LeChevallier et al (1988) showed that attached bacteria were 150 to 3000 times more resistant to hypochlorous acid than unattached cells. Anderl et al (2003) demonstrated that *Klebsiella pneumoniae* biofilms resisted killing during extended

exposure to ciprofloxacin and ampicillin even though these agents had penetrated the biofilms. Interestingly, as soon as microorganisms detached and dispersed from the biofilms they regained most of their susceptibility towards the antimicrobials (Lewis 2001; Anderl et al 2003).

2.2.2 Factors and mechanisms responsible for antimicrobial resistance in biofilms

The control of biofilm growth requires a thorough understanding of not only the actions and effects of antimicrobials on biofilms, but also of the mechanisms and developmental processes of biofilm formation and function. Several factors, processes or mechanisms for the ability of biofilm communities to survive antimicrobial treatments have been proposed and studied over the last two decades (Costerton et al 1987; Nichols 1989; Stewart 1996, Lewis 2001; Anderl et al 2003; Stewart 2003). The two general factors looked at first, were the possibility of diffusion limitation experienced within biofilms (Costerton et al 1987; Stewart 1996), and slow growth rate of cell within the deeper sections of the biofilm caused by nutrient limitation (Brown et al 1988; Nichols 1989).

The inhibition of antimicrobial penetration or the deactivation of antimicrobials were observed due to the presence of an EPS matrix acting as a diffusion barrier (Stewart 1996; Stewart 2003). The structure and nature of the EPS matrix determines the diffusion or solute transport processes within biofilms (Stewart 2003). Yet, dependent on the nature of the antimicrobial applied, substances like ampicillin were found throughout the biofilm structure without killing the bacteria (Anderl et al 2003). Planktonic cells in stationary growth phase or with a slow growth rate also showed increased resistance towards antimicrobials, and at times to a higher degree than fast growing biofilm cells (Spoering and Lewis 2001). This observation has lead to questioning the validity of whether biofilms were indeed less susceptible than planktonic cells (Spoering and Lewis 2001) to antibiotics and biocides.

Other mechanisms, including intrinsic and acquired resistance by mutations or the transfer of genetic material (Mah and O'Toole 2001), bacterial communication and interactions such as quorum sensing (Davies et al 1998; Whiteley et al 2000), as well as the presence of multiple drug resistant operons (Maira-Litran et al 2000) and efflux pumps (Maira-Litran et al 2000), have been proposed to be involved in the development of biofilm resistance. Lewis (2001) proposed another hypothesis for explaining biofilm resistance in the form of persister cells.

The observations and research results of the last decade showed the validity of most or even all of the proposed hypotheses, but again in most or all cases they have been found not to be the sole reason or mechanism contributing to the resistance phenomenon of biofilms (Allison et al 2000). In general, resistance cannot be explained by only one mechanism, as has been clearly seen earlier in the study of this phenomenon (Nichols 1989; Allison et al 2000).

Stewart (2003) discussed the influence of the diffusion process on the biology and chemistry of biofilm growth. He indicated that: 1) diffusion is predominantly a solute transport mechanism within cell aggregates, 2) most non-reacting substances have a similar diffusion rate as water, 3) diffusion leads to gradients in concentrations of substances and therefore to gradients in cell physiology and, 4) even though water channels within a biofilm carry substances in an out of the biofilm, they do not ensure that these substances reach the inside of cell aggregates.

Microorganisms have the ability to rapidly respond to their immediate environment, by changing their physiological processes, which could lead to a change in phenotype. Stressed cells may change the proteins that make up their cells walls, thereby becoming less permeable. Most toxic substances must enter the cell to be harmful, therefore they first have to cross the outer layers of a cell, which may form a permeability barrier-reducing uptake of substances (Brown et al 1988; McDonnell and Russell 1999).

Nutrient type and availability has a profound influence on the metabolic activity of microorganisms is the environment. This is valid for any living organism, simple or complex. It is therefore worth considering the potential influence of nutrients on resistance in biofilms. Growth rate, and more indirectly, diffusion and quorum sensing in biofilms are influenced by the way microorganisms respond to the nutrients in their immediate environment.

2.3 INFLUENCE OF NUTRIENT CONDITIONS ON SELECTED FACTORS INFLUENCING ANTIMICROBIAL SUSCEPTIBILITY

The nature of the nutrient sources available to the biofilm community has a significant impact on biofilm composition, architectural heterogeneity, physicochemistry, biofilm development and species diversity (Wolfaardt et al 1994a; Moller et al 1997). Therefore, nutrient conditions could affect most of the previously mentioned factors and mechanisms contributing to antimicrobial resistance, namely the production and existence of an EPS matrix as a transport limiting structure, growth rate, changes in phenotype and adaptive mechanisms. Nutrient conditions may also have an influence on the need or ability of microbial cells to grow in planktonic or sessile conditions, partly due to the activity levels of genes responsible for attachment or detachment (Dewanti and Wong 1995).

Most biofilm communities develop a permeable matrix via the production and excretion of EPS. The nature and abundance of EPS is partly dependent on the nutrient conditions, therefore the latter also affects the structure and composition of this matrix. The EPS matrix largely contributes to the overall biofilm composition, structure and dynamics. This includes the possibility and degree of transport limitations of nutrients and other compounds within zones in the biofilm, causing starvation and slow growth (Xu et al 1998) or the accumulation of toxic substances. Gradients in nutrient concentrations may lead to corresponding gradients in growth rate or other physiological activities (Xu et al 2000). Cells are known to change their phenotypic state and physiological activities in response to their immediate environment, especially when experiencing starvation or when exposed to toxic substances. Slow growth could cause expression of dormant or starvation phenotypes, which in turn often over express non-specific defenses such as shock proteins, multi-drug efflux pumps and extracellular polymers (Allison et al 2000).

2.3.1 EPS production

The load of EPS production is dependent on the microbial strain, culture conditions and the medium compositions (Looitjesteijn et al 1999). Looitjesteijn et al (1999) showed that exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* was regulated by the carbon (sugar) source. A marked difference in the production capacity of sugar nucleotides

(EPS precursors) was observed when *L. lactis* was grown on glucose than when grown on fructose. Glucose as a nutrient source thus increased the production of EPS by this organism.

Dewanti and Wong (1995) were interested in the influence of culturing conditions on biofilm formation by *Escherichia coli* O157: H7. They observed that minimal salts medium (MSM), containing specific carbon sources promoted biofilm formation with a thick EPS matrix and shorter cells. Glucose as carbon source showed to be the best substrate for stable biofilm formation by *E. coli* O157:H7. In contrast, in tryptone soy broth (TSB) medium *E. coli* showed poor biofilm formation lacking an EPS matrix and easy detachment of cells occurred. Changing the nutrient conditions from TSB to MSM caused an increase in attached cells and presence of EPS, while a change from MSM to TSB caused cell numbers and quantity of EPS to decrease. These observations are in contrast with those of Christensen and Characklis (1990), who found that higher nutrient concentrations and loading rates tend to produce thicker and denser biofilms than low nutrient concentrations. The presence of proteins in the TSB medium could be a reason for poor cell attachment and EPS production, which stands in contrast with the observation made by Bonet et al (1993). These authors studied the effect that nutrients have on exopolysaccharide production and surface properties of the bacterium *Aeromonas salmonicida* grown in planktonic populations. Two types of exopolysaccharides were defined, those that were detached from cells, and cell-bound capsular polysaccharides (CPS). It was found that the bacterium did not produce CPS or non cell-bound polysaccharides when glucose, phosphates, magnesium chloride or trace minerals were absent from the medium. EPS and CPS production depended on the initial concentrations of carbon or nitrogen sources and not on the C:N ratio. Capsular production influenced the cell surface properties and thus was associated with increased cell hydrophilicity and auto-agglutination. An increase in peptone concentrations caused an increase in EPS production. Furthermore, starvation conditions appear to increase the production of extracellular polysaccharides (Wrangstadh et al 1990; Schlictman et al 1994)

EPS production typically requires a large amount of energy, which may in turn lead to a decrease in growth rates by the producer. This was observed by the reduced growth of EPS producing or capsule forming species while the growth of non-producing species was stimulated due to a difference in energy cost (Kreft and Wimpenny 2001). Again, the production of EPS and CPS had a dramatic influence on the structure of the biofilm (Kreft and Wimpenny 2001). Evans et al (1991) observed the difference in the susceptibility of a

mucoid and a non-mucoid *Pseudomonas aeruginosa* strain to ciprofloxacin with respect to their growth rate and whether cells were planktonic or sessile. Antimicrobial susceptibility by *Pseudomonas* strain in suspension (grown in chemostat) was directly related to the growth rate, with no difference between mucoid and non-mucoid strains. In contrast, the non-mucoid *Pseudomonas* strain had a reduced growth rate dependency when growing in a biofilm, being more sensitive at slow growth rates and more resistant at high growth rates than their planktonic counterparts. The mucoid *Pseudomonas* strain growing in a biofilm were unaffected by the antibiotic, while the organisms resuspended from the biofilm had similar growth rate dependency as their planktonic counterparts. Bonet et al (1993) observed that the production of EPS and CPS by the bacterium *Aeromonas salmonicida* grown in batch culture, started only at the end of the logarithmic growth phase.

2.3.2 Biofilm architecture

Moller et al (1997) observed that a biofilm community grown on 2,4,6-trichlorobezoic acid (2,4,6-TCB) as sole carbon and energy source developed a characteristic architecture (basal layer of cells and conspicuous mounds of bacterial cells and polymer). When the carbon source was exchanged with a labile, non-chlorinated carbon source (TSB), a change in biofilm architecture (loss of mound structure and formation of a more homogenous biofilm) was observed. Another example of the effect caused by the growth medium was observed by Wolfaardt et al (1994a), where diclofop methyl-degrading bacteria formed thinner biofilms when grown on a TSB medium (labile carbon source) than when grown on a medium containing diclofop as the only carbon source. It was observed that TSB-grown biofilms were less variable in terms of thickness, spatial orientation of cells, cell density and cell morphology than diclofop grown biofilms. Karthikeyan et al (2001) studied and assessed the adaptation of bacterial communities to environmental transitions from labile to refractory substrates. The biofilm thickness and extent decreased, and the architecture of the biofilms changed, when the substrates were switched from labile to refractory.

A difference in diffusion rates was measured in biofilms grown with sucrose as opposed to those grown in the absence of sucrose as carbon source. Sucrose supplemented biofilms had diffusion rates close to those in free solutions (less hindered), while sucrose starved biofilms had slower diffusion rates, depending on the dextran molecular mass and location within the biofilm (Birmingham et al 1995).

2.3.3 Biofilm heterogeneity

Heterogeneity was proposed as a possible explanation for bacterial resistance against antimicrobials (Korber et al 1997; Xu et al 1998; Xu et al 2000). A degree of heterogeneity has been found in pure and mixed-species biofilms in terms of channels, pores, detectable microcolonies, distinctive cellular arrangements, regions of high cell or polymer densities, metabolic conditions or chemical gradients (Korber et al 1997). Xu et al (1998) hypothesized that the physiological status varied spatially within a biofilm community (even within the mono-species biofilm) and that the physiological activity was controlled by the oxygen availability. They observed that the oxygen penetration profile changed with depth in the biofilm. This also agrees with the assumption that with increasing depth the chemical composition changes within a biofilm. Corresponding to the observations mentioned above it was observed that the activity and viability are maximal near the biofilm/bulk fluid interface and decreasing with depth into the biofilm or microcolony (Huang et al 1998; Xu et al 2000; Whiteley et al 2001).

Physiological heterogeneity within a biofilm could be caused by altered physiological mechanisms, or by growth that is controlled by nutrient availability or product inhibition. Nutrient limitation, according to Xu et al (2000), is not the only cause for spatial heterogeneity, for dead cells may act as a nutrient source for the surviving cells. Other mechanisms may also be responsible for spatial heterogeneity. These include: 1) cell to cell signaling that is responsible for switching of cells into a protective phenotypic state or dormancy, and 2) the presence of cells that do not reproduce, but maintain metabolic activity, to act as a shield for the surrounding cells from unfavorable nutrient exposure, thereby promoting resistance towards antimicrobials (Xu et al 2000).

Huang et al (1998) observed that a distinct spatial pattern of alkaline phosphatase (Apase) expression developed in bacterial colonies and pure culture biofilms (*P. aeruginosa* and *K. pneumoniae*) in response to phosphate starvation. The patterns of expression differed between the two organisms. They hypothesized that these patterns and their differences could be explained by the local availability of carbon and energy source (nutrient) or electron acceptor (oxygen) to the bacteria.

2.3.4 Growth rate

Microbial populations and communities demonstrate asynchronous growth, thus consisting of organisms at all stages of the division/ reproduction cycle. At any time, presuming balanced growth, a constant proportion is at any given stage of division (Gilbert et al 1990) and during division cycles enzyme levels undergo a series of ordered changes (Mitchison 1969). Unstable enzymes result in peaks of activity, where the enzyme degradation rate is more related to temperature and pH than to specific growth rate. The faster the division rate becomes, the more frequent the biosynthetic period and the higher the mean level of activity in a heterogeneous population. Therefore, if enzyme activity affects antimicrobial susceptibility, then the level of drug susceptibility expressed by asynchronous populations will not be homologous throughout the biofilm population.

James et al (1995) observed that attached stationary-phase *Acinetobacter* cells changed from coccoid to a bacillar morphology when supplemented with a high-nutrient medium. The change from coccoid to bacillar was accompanied by a decrease in attachment stability (detachment from the biofilm). Under starvation conditions, the morphology transition was reversed and attachment stability increased, again forming firmly attached tight packed microcolonies on the surface. A similar relationship was observed between growth rate (determined from increase in cell area coverage) and transition.

According to Lewis (2000), the rate of killing is proportional to the rate of growth. The resistance of planktonic *Samonella typhimurium* to membrane permealizing antibiotics was induced during stationary and starvation phase (McLoed and Spector 1996). This correlates with the statement that slow growth is a factor in increased resistance of stationary planktonic cells to killing (Lewis 2000). McLoed and Spector (1996) observed that planktonic *S. typhimurium* developed resistance, induced by starvation and stationary growth phase, against membrane-permealizing antimicrobial agents such as the peptide polymyxin B. Cells appeared to have developed greater resistance under phosphate starvation than under carbon and nitrogen starvation. From this the authors postulated that resistance was influenced by the time of exposure and growth conditions, which in turn influenced the growth rate. In contrast, Desai et al (1998) observed a progressive increase in resistance during the exponential growth phase of planktonic and biofilm cultures of *Burkholderia cepacia*. The authors found that the bacteria growing at the same growth rate might differ in their resistance to antimicrobial agents. Both, planktonic and biofilm cells showed increased resistance

towards antimicrobial treatment with every new generation. This has lead to the proposal that the growth rate was not the key factor for developing resistance, but that the growth phase and mode of growth could have a greater impact on the degree of resistance to killing (Desai et al 1998). The pattern of resistance observed could also be due to the consumption rate of nutrients and the density (Mah and O'Toole 2001) of the biofilm population.

It is assumed that the spatial heterogeneity of growth within a biofilm could be a crucial factor in determining the susceptibility to growth rate-dependent antimicrobial agents (Xu et al 1998; Xu et al 2000). So far it has been argued that an increase in resistance is partly due to the transition from exponential to slow or no growth. In contrast, Desai et al (1998) concluded that other factors than growth rate might be responsible for resistance, while slow growth may add additional protection.

Slow growth is generally partly due to nutrient limitation, but could also be due to a stress response (Foley et al 1999; Mah and O'Toole 2001). Stress response results in physiological changes to protect cells from various environmental stresses. The resistance observed during starvation or unfavorable environmental conditions, could primarily be the result of a general stress response, and thus to a lesser degree be an adaptation to nutrient depletion or starvation. The general stress response (GSR), occurring during the stationary phase of non-sporulating environmental bacteria, is thought to result in cells resistant towards numerous chemical and physical stresses (Foley et al 1999). The GSR may result into a sub-population of cells contributing to the characteristic antimicrobial resistance. Foley et al (1999) proposed that an early general stress response in a biofilm, induced by the accumulation of density-dependent signals, could play an important role in the resistance of biofilms.

Lewis (2000) proposed and discussed the existence of persister cells as another idea. Persister cells are proposed not to be mutants and do not represent a special stage in the cell cycle, thus are not cells in dormancy (state of no growth). Little is known about this proposed phenomenon of persister cell.

2.4 ANALYSIS OF MICROBIAL DIVERSITY AND STRUCTURE IN MICROBIAL COMMUNITIES

Accurate fingerprinting of microbial community diversity and structure requires the knowledge of species richness (number of species within the community), species evenness (size of species population within a community) and the physiological role of each species in the environment and their interactions(s) in the community (Amann et al 1995; Liu et al 1997).

2.4.1 Community ecology

Ecology is the study of interactions of organisms with each other and their physical-chemical environment. The ecology of biofilms or any other microhabitat is influenced and maintained by abiotic factors (e.g. nutrient composition, pH, temperature) and the dominant inhabitants. Flow systems are increasingly being used for biofilm ecological studies, which consider amongst others physiological activity levels, growth rates and population dynamics.

A population can be defined as all the individuals belonging to a single species within a microhabitat, while a community consists of all the populations within that microhabitat. Community ecology is generally based on the succession theory. Primary succession occurs when a new surface or microhabitat that has not been conditioned by organisms, is colonized by organisms, which have the ability to survive and grow under these pristine conditions. These organisms are called pioneer populations. Ideal pioneer populations are generally those that either are adapted to very specific environmental conditions (e.g., oligiotrophic conditions) or have the ability to grow within a wide range of different conditions. An example is the ability to attach to bare surfaces that may enable subsequent biofilm development. Pioneer populations change the environment around them, conditioning it for other organisms to be able to establish. A pioneer population often does not remain the dominant population as other organisms contribute to the changing environmental conditions and as a result the pioneer population could be replaced. During succession, a community undergoes consecutive changes until finally there is a climax community that is at its most diverse state (Mader 1996). A climax community survives as long as the abiotic factors allow it. Secondary succession is observed when a climax community that has been disturbed returns to a state of increased diversity again (Mader 1996).

Could biofilms be seen as small ecosystems of their own? Biofilms can be viewed as small independent systems forming or being part of a functional ecosystem. The activity and dynamics of the biofilm community depend on the growth and metabolism (substrate consumption, reproduction, synthesis of EPS) of each population present in the community. Biofilms are proposed to reach a steady state due to processes reducing or removing (sloughing or deliberate detachment of organisms) biomass from biofilm surfaces.

Succession of biofilm populations seems to depend on growth rate (Banks and Bryers 1991) and allocation of energy (growth or maintenance). The succession theory according to Garland et al (2001) predicts that early succession will be dominated by populations with broad niche width, rapid growth and high investment in reproduction (opportunistic organisms). Dominance of population can shift during later succession to species with narrow niche width, slower growth and lower investment in reproduction (equilibrium organisms). Garland et al (2001) distinguished between non-culturable type and non-culturable state of organisms, both having distinct microbial community dynamics regarding changes in the ratios of culturable and non-culturable organisms. The authors predicted that succession could affect culturability of microorganisms. Based on the assumption that the ability to grow on nonselective medium is an indication of energy directed into growth, they proposed that the microbial community in the early succession consists of a higher proportion of culturable types. Later in succession a shift to non-culturable types occurred, which was confirmed by the decrease in culturable organisms, thus less energy was direct into growth and more energy into maintenance. The change was not due to cell death, but because of a shift in community composition, by clear indication of cell activity (reduction of CTC) and changes in community profiles obtained by analyzing terminal restriction fragment length polymorphism (T-RFLP) (Garland et al 2001).

2.4.2 Spatial arrangement and composition of biofilm communities

Banks and Bryers (1991) found that the establishment of bacterial species within a biofilm composed of another species depended on the relative growth rate of the organism. When both bacterial species were deposited simultaneously, the faster growing organism became dominant. The slower growing organism was not replaced, but remained established in the biofilm and continued to increase in number over time. Biofilm removal rates showed that

the amount of cells of both species detected in the effluent changed when comparing pure culture biofilms to mixed culture biofilms. The difference could be due to the spatial distribution of the species, with the slower growing species at the deeper layers of the biofilm and the faster growing species dominating the upper layers close to the water interface. This suggests that in a mixed-species biofilm the rate of species removal may not correlate to the concentration of species found within the biofilm due to non-uniform distribution (Banks and Bryers 1991).

According to Pedersen (1990) the initial bacterial composition had only a limited influence on the mature biofilm community composition since the initial attachment of microorganisms to surfaces was dominated by growth, product formation and debris entrapment. Rickard et al (2003) proposed that in addition to enhancing biofilm development, coaggregation between microorganisms also influenced the microbial diversity of freshwater biofilms. It has been shown that coaggregation was mediated by growth-phase-dependent lectin-saccharide interactions, which are found to be optimal in stationary phase cultures (Rickard et al 2000). Rickard and coworkers (2003) also found that species diversity and coaggregation interactions were greater in biofilms than in planktonic populations and that the presence of sugars could partially or completely inhibit coaggregation. Whiteley et al (2001) found that the dominant planktonic and biofilm species differed when 20 bacterial strains were co-cultured in a glucose limited chemostat and that biofilm density increased with increasing species diversity. *Pseudomonas putida* population size was greater and *Vogesella indigofera* smaller in binary than in pure culture biofilms.

2.4.3 Effect of antimicrobial treatment on population dynamics

Norton and LeChevallier (2000) observed a dramatic shift in bacterial community composition to a predominant gram negative biofilm population and a predominant gram positive planktonic population when adding free chlorine to conventional and biological treated water. The combination of free chlorination and low nutrient levels resulted in selective pressures that permitted only a few of the bacterial populations from the biofilm community to survive (Norton and LeChevallier 2000). It has also been observed that the presence of a betadine-resistant organism enhanced the survival of a betadine-sensitive organism when grown in a binary biofilm (Whiteley et al 2001).

2.4.4 Viable, but not culturable microorganisms

Microbiologists have known for generations that conventional culture-dependent techniques (viable plate count or most-probable-number techniques) were inadequate for quantification of active cells and in representing environmental community diversity and structures due to cultivation selective pressure (Amann et al 1995). Traditionally, cultivation methods have been and are still used for determining the presence of microbial life as well as the life or death state of microorganisms in samples of interest. A large fraction of microorganisms has been discovered to be non-culturable (85% - 99.99%) and thus undetected by culture dependent methods (Ward et al 1990). Rockabrand et al (1999) stated that the differences between total and culturable cell counts, and the diversity of 16S rRNA sequences found in uncultivated samples compared to culture collections indicate that most bacteria are non-culturable. It has been frequently observed that direct microscopic counts exceeded plate counts of cultivable organisms by several orders of magnitude, especially in oligotrophic aquatic habitats. Staley and Konopka (1985) named and described this phenomenon the "great plate count anomaly". It is thus clear that cultivation may cause changes in original environmental parameters and therefore changes in the original community structure.

Non-culturable microorganisms can be divided into two groups, those that are culturable, but have lost their ability to reproduce and those that to date have not been cultured. The loss of plating or growing efficiency has traditionally been assumed to represent microbial death as a consequence of starvation-induced deterioration (Schlegel 1986). The confirmation of the second group of non-culturable microorganisms provided a rationale for the development of other hypotheses for explaining this phenomenon of the "great plate count anomaly". Three different models have so far been proposed. The first model is based on the traditional opinion of stochastic deterioration. The two other hypotheses are based on the assumption that genetically programmed survival strategies exist that activate the non-culturability state, either by being an adaptive pathway generating dormant phenotypes (viable but non-culturable) or by entering programmed cell death (Nyström 2003).

Nyström (2003) agued in favor for the original theory of starvation induced non-culturability leading to cell death when adverse conditions are maintained for too long. The viable but non-culturable state due to starvation involves the activation of protein synthesis necessary for preventing cell death occurring too early. Increased oxidation of proteins was observed during stages of no-growth when either the sigma factor σ^S (responsible for the binding and

directing of RNA polymerase) or other transcriptional controlling genes were lacking (Dukan and Nyström 1998, Yasuda et al 1999). It was suggested that an increased demand in oxygen management is needed in cells under starvation (Nyström 2003). Several cell processes seem to be possible targets for stasis-induced damage. Unculturability of cells due to starvation response could be reversed should specific conditions occur, and thus rescue cells from death (Pedersen et al 2002; Nyström 2003). According to Nyström's reasoning the hypothesis of programmed cell death is refuted by showing that the toxin-antitoxin mechanism of the TA loci has been shown to be beneficial to the cells (Gerdes 2000) instead of killing the cells. The hypothesis of non-culturability, being an induced genetically controlled survival mechanism, falls short due to the lack of proof of viable but non-culturable (VBNC) resuscitation, the present absence of VBNC mutants, and the lack of information on genes involved in this particular genetically control pathway (Nyström 2003).

The term viable but non-culturable has been developed to describe non-growing but metabolically active and intact microbial cells observed during unfavorable environmental conditions e.g. nutrient starvation (Lowder et al 2000), low or high temperatures (Gunasekera et al 2002) or presence of toxic substances (Rockabrand et al 1999). Thus, microorganisms that have lost their culturability due to injury caused by unfavorable environmental conditions e.g. heat or biocide treatment have also been described as viable but non-culturable (Gunasekaera et al 2002). More is known about the early stationary phase, than what is known of the physiology of the true metabolic dormancy and the VBNC state, and the relationship between the two latter states (Rockabrand et al 1999).

The lack of proof for VBNC resuscitation stands in contrast with the results obtained by Rockabrand et al (1999), who were able to resuscitate close to a 100% of the initial coliform community after chlorine treatment. This coliform community lost its culturability after chlorine treatment. According to the authors, the regrowth of coliform could not have been due to rare surviving cells, for most stationary phase cells (shown by high levels of the Dps protein) reverted to actively growing cells (shown by high levels of the Fis protein), visualized by changes in protein profiles, when supplemented with nutrients. No significant numbers of residual dormant cells during the recovery phase were found. Heim and coworkers (2002) studied the protein expression patterns of exponentially growing, starved and VBNC of *Enterobacter faecalis*. They found that the VBNC cells constitute a distinct

physiological state based on the protein profile difference of VBNC from those of either starved or exponentially growing cells.

Different techniques and methods have been developed and applied to study viable but non-culturable microorganisms. These techniques are based either on the membrane potential, membrane integrity or expression of metabolic activity genes. Vital staining methods (Gunasekera et al 2002) rely on the assumption that cells with impaired cell walls and membranes are non-viable. Gene expression methods make use of metabolic markers like the green fluorescent protein (GFP) (Sternberg et al 1999; Lowder et al 2000; Gunasekera et al 2003) or a combination of gene products representing different growth states (Rockabrand et al 1999; Heim et al 2002) to indicate metabolic activity.

Could VBNC be a physiological state found in biofilm cells? It was observed by Sternberg et al (1999) that bacteria grown in a biofilm reduced their metabolic activity after some time even though enough nutrients were still available. A short-lived *gfp*-gene carrying plasmid, transformed into a *Pseudomonas* strain, was used as an indicator of metabolic activity. Fluorescent counts decreased after some time in the flow-chamber biofilms, and increased again for a short period when a different carbon source was added, before decreasing again. It therefore, could be of interest to confirm whether these low metabolic active cells also exhibit a non-culturable state.

2.4.5 Culture-independent methods for microbial diversity analysis

Traditionally, microbial taxonomy depended on the culturability of microorganism, but the apparent inadequacy lead to the use of genospecies on the basis of a DNA-DNA similarity (Schleifer and Stackebrandt 1983, Woese 1987). Culture-independent molecular techniques, based on the genotype of microorganisms, revealed a formerly unknown diversity of microorganisms. DNA-DNA re-association studies on samples directly isolated from soil indicated a complexity of different sizes of bacterial genomes (Szewzyk et al 1994). Interestingly, according to Amann et al (1995), the recovery of unknown sequences came to many as a surprise, indicating that the sequences deposited on the DNA database (NCBI and Ribosomal database project) at that time (late 1980's) only encompassed a small percentage of the known species and did not represent the natural diversity of microorganisms.

2.4.6 History of molecular methods

More or less two decades ago the first applications of rRNA molecule analysis (Lane et al 1985; Stahl et al 1985) led to the discovery and development of several culture-independent molecular techniques to describe and study the diversity and structure of microbial communities in different environments. It started with the application of the 5S rRNA molecule, advancing to the larger 16S rRNA (±1500 base pairs) and 23S rRNA (±3000 base pairs) molecules, which supplied sufficient information for reliable phylogenetic analysis (Lane et al 1985; Amann et al 1995). Small subunits of ribosomal nucleotide acid molecules contain large sections of conserved sequences, which have been shown to be specific to groups of microorganisms (Woese 1987). Initially, DNA clone libraries in bacteriophage lambda (Schmidt et al 1991) were developed and constructed, but these were shown to be time consuming and laborious. The incorporation of PCR (polymerase chain reaction), firstly done by Giovannoni et al (1990), into gene libraries containing amplified 16S rRNA gene fragments provided a faster screening method. Community richness and evenness was based on the number of unique clones and relative frequency of various ribotypes. Therefore, other culture-independent molecular techniques were developed to avoid the construction of clone libraries. These techniques depend on the different characteristics of the 16S rRNA gene or functional marker genes (Tiedje et al 1999; Lueders and Friedrich 2003) of microorganisms, coupled to the phylogenetic framework (Woese 1987; Amann et al 1995).

2.4.7 Culture-independent molecular techniques

Culture-independent molecular techniques currently used for defining community diversity are based on DNA melting behavior [denaturing gradient gel electrophoresis (DGGE)], single strand DNA conformation (SSDC), the positions of restriction enzyme cutting sites [restriction fragment length polymorphism (RFLP) or amplified ribosomal DNA restriction analysis (ARDRA) and terminal restriction fragment length polymorphism (T-RFLP)] and conserved 16S DNA regions that are species or division specific as probes for *in situ* hybridization (FISH).

Denaturing gradient gel electrophoresis (DGGE) (Muyzer et al 1993, Muyzer 1999) depends on the melting behavior of DNA. DGGE is based on the electrophoretic separation of partial small-subunit rDNA fragment of the same length, but having different base pair composition within a linearly increasing gradient of denaturants. This method is limited by comparatively

insensitive staining techniques and the presence of a few dominant bands representing a highly complex microbial community. Therefore, it cannot be used for providing information concerning phylogenetic groups, as the band pattern of the community masks the band pattern of each phylogenetic group present in the community. Single strand DNA conformation (SSDC) separates PCR amplification products based on conformational differences of folded single-stranded products, which results into differences in electrophoretic mobility (Lee et al 1996). RFLP or ARDRA are based on 16S rDNA fragment length polymorphism (Moyer et al 1994, 1996) and was used for clone screening prior to sequencing (Pace et al 1986). Today, ARDRA is used for rapid comparison of rDNA's for detecting changes in communities.

2.4.7.1 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP is an extension of the RFLP / ARDRA techniques (Avaniss-Aghajani et al 1996; Liu et al 1997) and has become a useful tool for genetically fingerprinting the composition of environmental microbial communities. T-RFLP is a sensitive method to differentiate between microbial communities and for monitoring diversity, structure and dynamics of microbial populations (Liu et al 1997; Blackwood et al 2003; Lueders and Friedrich 2003). This method is often applied for comparing community diversity, based on the variation in position of restriction enzyme sites among isolated sequences, such as 16S rDNA. Among the strengths of T-RFLP is its speed and high sample throughput. This technique enables sample replication, prediction of terminal restriction fragment lengths (T-RF's), provision of numerical data and the comparison of predicted with observed T-RF's (Dunbar et al 2001). T-RFLP has been compared with DGGE and 16S rRNA gene cloning for effectiveness and consistency (Moeseneder et al 1999; Tiedje et al 1999; Dunbar et al 2000), which emphasized the use of T-RFLP.

Terminal restriction fragment length polymorphism is a PCR based technique. Extracted genomic DNA from a microbial community acts as a template for a specific chosen primer set. One or both of the primers used for the amplification are labeled with a fluorescent dye. After restriction enzyme digests of the amplicons, the different sizes and quantities of labeled terminal restriction fragments (T-RF's) are quantified with an automated gel or capillary DNA sequencer. A distinct microbial community fingerprint is obtained in the form of an

electropherogram in terms of fragment length and intensity (peak height or peak area) (Liu et al 1997).

Qualitative (presence or absence of distinct populations in sample) and especially quantitative (relative signal intensity) data interpretation requires careful and adequate controls. The choice of primer set and restriction enzymes used are vital for effective T-RFLP analysis (Dunbar et al 2001). Annealing efficiencies and the number of unique terminal fragments could be estimated beforehand using known 16S rDNA sequences found in the available databases (NCBI and ribosomal database project). The choice of the restriction enzymes to provide an optimal distribution of T-RF's is important, as for any given enzyme few T-RF's are truly specific for single species or members of a single genus (Liu et al 1997; Marsh 1999).

Current shortcomings of this technique are that 16S rRNA genes from phylogenetically related organisms could result in the formation of identically sized T-RF's (Avaniss-Aghjani et al 1996). It has been observed that in general, the average phylogenetic resolution for T-RF's profiles of natural environmental samples may be so low that phylogenetic deductions of community composition becomes not recommendable (Dunbar et al 2001). Another problem in terms of T-RF's is a possible difference in the predicted and observed T-RF's (Liu et al 1997; Clement et al 1998). This could be due to either the amount of *rrn* sequence variation among strains of the same species (States 1992) or due to incomplete restriction enzyme digestion, resulting in extra T-RF's. A third reason involves the discovery of pseudo-T-RF's formation caused by partially, single stranded PCR amplicons (Egert and Friedrich 2003). Extra T-RF's can lead to a false microbial diversity pattern, especially with pseudo-T-RF's, which do not remain constant in length and number, and could thus cause false assumptions made on their presence or absence. Another drawback is the lack of universal primer sets, which so far only represent a portion of the total species in the microbial world (Amann et al 1995; Brunk et al 1996).

The extraction of genomic DNA, cell lysis and PCR amplification could add a host of potential biases leading to a lower representation of community diversity and structure. PCR biases include preferential amplification of certain templates (von Wintzingerode et al 1997; Polz and Cavanaugh 1998; Marsh et al 2000), template re-annealing with increased PCR cycle numbers (Mathieu-Daude et al 1996; Suzuki et al 1998) and PCR annealing

temperatures (Clement et al 1998). Several studies evaluated the impact and presence of PCR bias influencing T-RFLP profiles (Liu et al 1997; von Wintzingerode et al 1997; Blackwood et al 2003). Due to potential PCR bias and largely unknown *rrn* copy numbers (number of rRNA gene operons) in microbial genomes, T-RFLP profiles do not reflect cell counts of natural microbial communities (Amann et al 1995). Very little is known whether relative gene frequencies of individual community members are adequately represented in post-PCR amplicon frequencies as shown by T-RFLP (Lueders and Friedrich 2003). It was shown by Lueders and Friedrich (2003) that in a defined template mixture of a four-member community, ribosomal DNA template-ratios were accurately recovered by PCR and T-RFLP analysis, while when using functional marker gene templates, the template ratios was not recovered. The degeneracy of the primers used for amplification of the functional gene was proposed as a possible reason for the inadequate recovery of template ratio.

These limitations may have an influence on whether the relative gene frequencies of individual community members are adequately represented in post-PCR amplicon frequencies as shown by T-RFLP (Lueders and Friedrich 2003). For successful application of T-RFLP in monitoring and analyzing complex environmental microbial communities, it is thus essential to have a thorough knowledge of its strengths and current limitations (Dunbar et al 2001).

2.4.7.2 Fluorescent *in situ* hybridization (FISH)

Molecular techniques based on PCR opened the door for culture-independent techniques (as mentioned above) to sensitive detection and identification of microorganisms, but most of these techniques lack the ability to provide information about morphology, number, and spatial distribution of microorganisms (Moter and Göbel 2000; Amann et al 2001) in environmental samples.

Fluorescent *in situ* hybridization (FISH) is a well-established molecular technique (Amann et al 1995) and has become an integral part of the rRNA-based approach to microbial phylogenetic (Amann et al 1990a) and ecological research (Olsen et al 1986). FISH has become a widely used tool for direct, cultivation-independent identification of microbial species (Amann et al 1995), in complex environmental samples due to its speed and sensitivity. Whole cell hybridization can be done on microorganisms found in a wide variety of environmental microhabitats and thus intact cells can be detected and studied.

The first to propose *in situ* hybridization (ISH) for counting and identification of microorganisms were Olsen et al (1986) and it was initially done with radioactively labeled oligonucleotides by Giovannoni et al (1988). In 1989, DeLong and coworkers (1989) used the first fluorescently monolabeled rRNA-trageted oligonucleotide probes for the detection of cells. Since then, fluorescent-labeled rRNA targeted olgionucleotide probes have become a common tool for the determination of cell morphology, abundance and spatial distribution of uncultured organisms or individual microbial cells in complex microbial communities (Amann et al 1990a; Amann et al 1995). FISH has been found to be compatible with direct count methods (Glöckner et al 1996; Maruyama and Sunamura 2000).

The rRNA molecules have become the main target for FISH analysis for several reasons. rRNA molecules are relatively stable (long living) and are found in all living organisms at high copy numbers. Importantly, they contain both variable and highly conserved sequence domains (Amann et al 1990b, 1995). These sequence domains carry sequence signatures that are unique to groups of microbes, ranging from whole phyla to individual species (Woese 1987). Because of the readily accessible public database that includes 16S rRNA sequences for most cultured microbial species, and a number of uncultured species (Maidak et al 2000; Van de Peer et al 2000; Maidak et al 2001), the 16S rRNA molecule has been the target for most FISH studies.

The design and specificity of the oligonucleotide probe used for hybridization and identification purposes is of utmost importance for the successful application of FISH. According to Fuchs et al (1998), one out of two newly designed probes failed, even when the positive controls demonstrated the presence of sufficient target rRNA and good permeation of the cells of interest. Therefore, every new probe should be tested on a reference microorganism before it is applied to environmental samples for quantification and *in situ* identification of microbial cells. It is vital to ensure that the fluorescence of the probe (probe-mediated fluorescence) is not affected by parameters like dissociation temperature, difference in ribosomal content, three-dimensional structure of the ribosome, autofluorescence or the quality of probe synthesis (Fuchs et al 1998). The quality or intensity of the signal from the probe depends strongly on the hybridization stringency conditions. Probes are generally between 15 to 30 nucleotides long and labeled at the 5'-end with a fluorescent dye, although

probes have been labeled with more than one fluorescent label to increase fluorescent intensities (Amann et al 1995; Moter and Göbel 2000).

The typical FISH protocol includes four to five main steps: 1) fixation and permeabilization of the sample, 2) if needed, preparation of the sample with specific pretreatment steps e.g. lysozyme, 3) hybridization with specific probes, 4) washing of the sample to remove unbound probes and ensure hybridization stringency, and 5) the mounting, detection of labeled cells by microscopy or flow cytometry, followed by the analysis of the results. The effective fixation of the cells is also crucial for optimum FISH results. Optimum cell fixation requires the effective permeabilization of the cells for the penetration of fluorescently labeled probes and also the protection of the rRNA from degeneration by endogenous RNAses. Two fixation approaches are available - precipitating agents (ethanol or methanol) or cross-linking agents (aldehydes) (Moter and Göbel 2000).

Problems and pitfalls of FISH were outlined by Amann et al (1995), include false positive results and false negative results (Amann 1995; Moter and Göbel 2000). False positive results were either autofluorescence and / or unspecific binding of probes. Autofluorescence could be due to the autofluorescence of microorganism themselves (e.g. *Pseudomonas* species) or from other factors like the growth media, background or fixation methods. As already mentioned above, the specificity of the probe influences the reliability and accuracy of the FISH technique. False negative results could be encountered due to insufficient probe penetration, higher order structure (folding) of the target or probe, low rRNA content and photobleaching.

Fluorescent *in situ* hybridization of whole cells has been applied to a great variety of fields and environments. Microbial communities in natural environments e.g. aquatic habitats, soils, or root surfaces, have been investigated. Microbial diversity in wastewater treatment plants are also identified and studied using this approach. FISH has been applied to study symbiotic bacteria, since most of these obligate microbial symbionts are still uncultured. The application of FISH in the medical field has been discussed by Moter and Göbel (2000), involving e.g. the studies of gastro-intestinal flora or the detection of pathogens. FISH can be used in combination with other techniques, increasing its reliability, e.g. DGGE (Fuchs et al 2000), microautoradiography (Lee et al 1999), micro-sensor techniques (Amann and Kuhl 1998), T-RFLP and traditional culturing techniques (Böckelmann et al 2000). According to

Amann et al (2001), FISH is a fast method compared to other molecular techniques, being the method of choice when exact cell numbers and cellular locations are needed. FISH combines the precision of molecular genetics with the visual information from the microscope (Moter and Göbel 2000).

CHAPTER 3:
MATERIALS AND METHODS

3.1 COMPARISON OF ABUNDANCE AND ANTIMICROBIAL SUSCEPTIBILITY OF BIOFILM AND PLANKTONIC MICROBIAL COMMUNITIES IN AN OPERATIONAL COOLING TOWER SYSTEM

3.1.1 Cooling tower system

The cooling tower system used for sampling was situated in an open-air courtyard on the campus of Stellenbosch University. The system provided easy access to industrially used water that was regularly treated with biocides for controlling corrosion and biofouling. As part of the routine maintenance of the system, biocides were added once a week for the four-week period of experimentation.

3.1.2 Biofilm development, sampling and planktonic cell counts

The LIVE/DEAD® *Bac*Light™ Viability kit (Molecular Probes Inc., Eugene, Oregon, USA) (referred as BacLight™ in all subsequent text) was used to monitor biofilm development and viability (Boulos et al 1999; Korber et al 1997). Planktonic populations were initially monitored using conventional plate count methods.

3.1.2.1 Biofilm analysis

Biofilm formation over time was observed by immersing and incubating a Perspex cassette, containing clean microscope glass slides (size 76 x 26 mm, thickness 1.0/1.2 mm, Blue Star), in the water collection tray of the water-cooling tower. Slides were removed daily from the container for 24 consecutive days. Each glass slide was rinsed with sterile tap water and stained for 15 min in the dark with 50 µl of BacLight™ viability probe, mixed according to the product information sheet [2.0 µl of component A (3.34 mM SYTO® 9 nucleic acid stain) and 2 µl of component B (20 mM propidium iodide) diluted in 1.0 ml of distilled water]. Biofilm regrowth was determined by adding clean slides for three days following each routine biocide treatment and sampled after 24 h, respectively. All collected glass slides were

analyzed and viewed with a dual-channel epi-fluorescence microscope (Nikon Eclipse E400) at 600 X magnification (10 x 60x1.4 oil Nikon lens). Between ten and twenty randomly chosen areas on each glass slide were used for statistical biofilm image analysis. Images were captured with a high performance CCD Camera (Model no. 4912-5010/0000) and analyzed with Scion Image software (Scion Corporation, USA, http://www.scioncorp.com). Biofilm viability and area coverage were determined by calculating the area coverage of viable cells (green fluorescence) as a percentage of total area coverage (area of both viable and non-viable cells).

3.1.2.2 Planktonic cell analysis

Water samples from the cooling tower were taken on a daily bases, simultaneously with collection of the glass slides. Each water sample was serially diluted in Ringer solution (2 tablets per liter, Merck) and spread plated on 3.0 g/l Tryptone Soy Broth (Biolab Diagnostics, Merck) agar plates (TSA) followed by incubation at 30 °C for 48 to 62 h. Lower concentrations of tryptone soy broth (TSB) were used to represent nutrient conditions found in oligiotrophic water systems. Colony forming units (CFU/ml) were counted to determine viable planktonic cell counts.

3.2 INFLUENCE OF HETEROGEINITY ON REQUIRED SAMPLE SIZE

3.2.1 Analysis of biofilm heterogeneity and sample size

Biofilm-covered glass slides and water samples were collected from the same system as explained in the previous Section 3.1.2. On each sampling day (day 4, 7 and 14, respectively), three glass slides were removed and placed into separate bottles containing 100 ml of water from the cooling tower system. Two of the three slides were treated for 5 h with 10 µl of a biocide stock solution, containing a mixture of glutaraldehyde and isothiazoline, in 100 ml cooling tower water, which had previously been added to the bottles. All three bottles containing glass slides were placed in the cooling tower water during the biocide treatment. After the incubation period, the glass slides were rinsed with sterile tap water and stained with 100 µl of BacLight^{TM} for 15 min. Unbound stain was removed with water and cover slips

were mounted on the slides with immersion oil (Type A, nd of 1.515, Nikon) for microscopic analysis. Each glass slide was divided into a grid of 60 identical fields of 5 mm by 5 mm. Each square was analyzed for spatial heterogeneity of biofilm viability and area coverage as described previously (see Section 3.1.2).

3.2.2 Analysis of planktonic cell viability and sample size

Three milliliters of cooling water from each of the three bottles were stained with 100 µl of BacLight™ for 20 min in the dark. The samples were vacuum filtered onto 0.22 µm polysulfone membrane filters (47mm Micron-PES, Osmonics Inc.) and covered with BacLight™ mounting oil (Molecular Probes) and a glass cover slip for further microscopic analysis (see Section 3.1.2). A minimum of thirty microscope images was randomly analyzed, for cell viability and abundance as described previously. The abundance of cells was expressed as percentage of total cell biomass covering the filter.

3.2.3 Statistical calculations for sample size of biofilm and planktonic analysis

The average glass slide size was 26 x 76 mm, which was divided into 15 x 5 mm squares each with an area of 25 mm^2. For area coverage analysis, between 60 and 70 images were examined. Each square represented one image and each image consisted of two pictures, one visualizing only red fluorescent cells (non-viable), and the other green fluorescent cells (viable).

The following two equations were applied to determine the number of images needed to obtain a statistically representative sample.

Number of images (n) = [1.96 x the standard deviation of the total images / 10% x mean of the total of the images]^2 [1]

Using this formula, the number of images was determined to obtain a 96% probability that less than 10% deviation from the mean had been reached. For this high level of probability the calculated (required) number of images proved to be not feasible, as >200 images were typically required, demonstrating the inherent variability of biofilm architecture.

A second statistical approach was consequently followed for estimating the optimal sample size (number of images needed) (Zar 1984). This involved repeated calculations of the coefficient of variation for the increasing number of images.

Coefficient of variation (c.v.) = Standard deviation / Mean of values [2]

By plotting the coefficient of variation against the cumulative sample size, the optimal sample size was obtained where the line through the data points asymptotes (FIG 3.2.3)

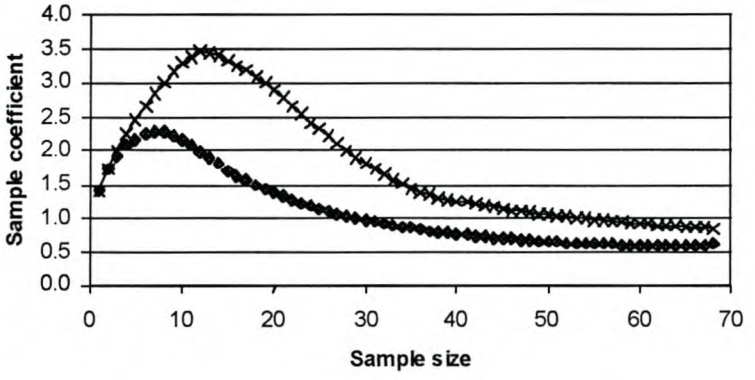


FIG 3.2.3 Graph obtained from equation [2] used for estimating representative sample size. In this case for both biofilm viability (X) and biofilm area coverage (diamond), the sample size was estimated to be between 60 and 70 images.

3.3 ISOLATION AND CHARACTERIZATION OF BACTERIAL ISOLATES FROM COOLING TOWER WATER

3.3.1 Isolation

Several bacterial species were isolated from the cooling tower water. These were to be subsequently used for the establishment of a defined biofilm microbial community. Five bacterial isolates were selected from spread plates obtained from the previous experiments (Section 3.1) and two from a biofilm samples obtained from a winery cooling system. The main criteria of isolate selection were distinct bacterial colony shape and/or coloration on TSA (3.0 g/l TSB plus bacteriological agar) plates. The different colony pigmentation or

margins were used to distinguish between the bacterial strains in subsequent biofilm population studies.

3.3.2 Identification by 16S rDNA sequencing

Bacterial genomic DNA from pure culture isolates was obtained as explained in Section 3.7.2.1. The 16S rRNA gene specific primers used to obtain 16S rDNA sequence of four selected isolates were fDD2, F1, F5, rPP2, R1, R3 and Univ (Table 3.3.1). Genomic DNA was used as template in a 50 µl PCR reaction containing 25 µl of PCR master mix (Promega), and 0.25 µM of each primer. The PCR reaction was carried out in a Perkin-Elmer, Gene Amp 2400 thermocycler. Thermal cycling conditions were 1 min denaturing at 95 °C followed by 25 cycles at 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 90 s, followed by a final extension at 72 °C for 2 min. The PCR product was purified using the GFX PCR DNA and Gel band extraction Kit (Amersham, Biosciences). Sequencing of the PCR product was done on an ABI 3100 Genetic Analyzer. Sequence fragments were assembled using the PC-based software of DNAMAN (Version 4.1) software package from Lynnon Biosoft, and BioEdit Sequence Alignment Editor (Hall 1999). Comparison searches of the assembled 16S rDNA fragments were preformed using the gapped-BLAST program at the National Center for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/BLAST/] (Altschul et al 1997).

Table 3.3.1 16S rDNA specific PCR primers used for amplification and sequencing of bacterial isolates.

Primer	Sequence	References
fDD2	5'-CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG-3'	Rawlings (1995)
rPP2	5'-CCAAGCTTCTAGACGGITACCTTGTTACGACTT-3'	Rawlings (1995)
Univ	5'-CCGGATCCGTCGACGTGCCAGCIGCCGCGGTAA-3'	D.E. Rawlings
R1	5'-GTATTACCGCGGCTGCTGGCAC-3'	D.E. Rawlings
F1	5'-AGAGTTTGATCITGGCTCAG-3'	D.E. Rawlings
F5	5'-GCATGGITGTCGTCAGCTCGTG-3'	D.E. Rawlings
R3	5'-CACGAGCTGACGACAICCATGC-3'	D.E. Rawlings

3.3.3 Specific growth rates in batch cultures

The specific growth rate for each of the selected isolates (CT01, CT03, CT04 and CT07), growing in different nutrient conditions was calculated from growth curves performed at room temperature (23°C). Nutrient conditions were 3.0 g/l Glucose, minimal salt medium (MSM) supplemented with 1.0 g/l Glucose and MSM + 0.1 g/l Glucose (see Section 3.5.2), respectively.

Pure cultures were inoculated in 10 ml TSB (3.0 g/l) and incubated at 30 °C overnight (18 h). One ml of overnight culture was centrifuged in a bench top centrifuge (Biofuge 13, Heraeus Instruments) at 13000 rpm for 5 min and the pellet was resuspended in 1.0 ml of the respective nutrient broth. Spectrophotometric readings were taken at 600nm every 1 to 2 h of 100 ml of respective nutrient broth culture that had been inoculated with 100µl of the resuspended overnight isolate culture.

3.3.4 Biofilm formation rate (BFR)

Attachment and biofilm formation rates of pure and multi-species biofilms were determined using an optical large area photometer (OLAPH; an experimental development). The large detection area enabled the detection of very thin biofilms. Forward scattered light of the biofilm was measured by a photo-cell (maximum of 900 nm). Data was captured at regular intervals through computer interfacing and Labview software and stored in an Excel spreadsheet.

A reservoir containing nutrient broth (3.0 g/l TSB) was connected via a peristaltic pump (Watson Marlow 250S) with silicon tubing (diameter of 1.3 mm x 3.0 mm) to the flow cell specifically designed for biofilm studies with total area coverage of 15 cm^2, and a total volume of 7.5 cm^3. The flow cell and the silicon tubing were sterilized with 3.5 % sodium hypochlorite for 2 h and then rinsed with nutrient broth for 2 h to remove all the remaining sodium hypochlorite under continuous flow conditions. No-flow condition was maintained for 1 to 2 h after inoculating (injecting) the flow cell with 2.0 ml of an 18-h-old overnight culture (see Section 3.3.3). The overnight culture was either a pure culture of 1 of the 4 isolates or a mixture of the 4 isolates. A constant flow rate of 19 ml/h, resulting in a laminar flow rate of 12.7 cm/h, was maintained for the duration of the experiment.

3.3.5 Interactions between isolates

Four bacterial isolates (CT01, CT03, CT04 and CT08) were tested for the potential of inhibiting each other's growth when cultivated together in a community. The bacterial isolates were cross-streaked on TSA plates (3.0 g/l TSB) in duplicate and incubated at 30 °C as well as room temperature. After 2 to 3 days, the plates were inspected for possible growth inhibition zones. This experiment was performed to confirm that the test organisms did not negatively influence each other when co-cultured.

3.4 DEVELOPMENT AND BEHAVIOR OF A DEFINED MIXED SPECIES BIOFILM COMMUNITY

In order to minimize the variables experienced in the cooling tower system, the subsequent experiments were conducted under controlled laboratory conditions.

3.4.1 Experimental set-up of flow cell microcosms

An eight-channel flow cell (Wolfaardt et al 1994a) was connected via a peristaltic pump and silicon tubing (Watson Marlow 250S) to a reservoir containing sterile medium (inner tube diameter = 1.3 mm). Each flow channel had a total area of 1.206 cm^2 and a total volume of 0.265 cm^3 (length = 3.10 cm, width = 0.4 cm, height = 0.22 cm). A glass cover slip acted as substratum for biofilm attachment. The flow cell system was sterilized with 3.5 % sodium hypochlorite under continues flow for 2 - 3 h and rinsed with sterile medium for a further 1 - 2 h. The flow rate for all flow cell experiments was kept at 5.5 ml/h for the duration of the experiment except during microscopy analysis when flow was stopped. This resulted in a laminar flow of 68.80 cm/h and a dilution rate of 20.75 h^{-1} [34 times higher than the μ_{max} (0.6 h^{-1}) of the fastest growing bacterial isolate]. The flow channel effluent was collected separately in a waste flask.

All flow channels were treated identically e.g. same inoculum, flow rate and nutrient conditions. It was therefore assumed that all flow channels contained identical biofilm communities. Biofilm development and behavior was monitored over a period of 21 days, by sacrificing and analyzing one flow channel at the time, on days 1, 3, 7, 18 and 21. Fluorescent staining prevented repeated analysis of the same flow channels.

3.4.2 Cultivation of biofilms

The initial biofilm community consisted of seven bacterial isolates (Table 4.3.1). Bacterial isolates were inoculated separately from frozen (-80 °C) or streak cultures into 10 ml TSB (3.0 g/l) and incubated for 18 h at 30 °C on a rotating wheel. The inoculum consisted of a 2.0 ml mixture of each of the different isolates. To enhance initial attachment, pump flow was only resumed 1 h after 0.2 ml of inoculum was injected into each test flow channel.

3.4.3 Biofilm analysis

For sampling and analysis, flow channels were injected with 0.2 ml BacLight™ solution (Section 3.1.2.1), while the flow of nutrient medium was stopped. After 15 min, the stain was washed out by turning the pump on for another 5 min. Fluorescently stained flow channels were visualized and analyzed using an epi-fluorescent microscope. Images were captured by using a high performance CCD Camera (Model no. 4912-5010/0000) and analyzed with Scion Image (Scion Corporation, http://www.scioncorp.com) software. Viability and total area covered by biofilms were determined at between 30 - 50 fields, in each case along three parallel sections over the length of the flow channel.

3.4.4 Analysis of planktonic cells

Flow channel effluent was collected within 30 min prior to fluorescent staining for the determination of planktonic cell viability (CFU/ml and fluorescent viability staining) and abundance (optical density and total cell area coverage on a filter). One ml of the effluent sample was stained with 50 µl BacLight™ solution. After at least 1 h, the stained sample was vacuum-filtered onto a black polycarbonate 0.22 µm filter (Osmonics Inc. Poretics) and analyzed by fluorescent microcopy. Twenty to thirty images were randomly selected per filter, and viability and area coverage analyses conducted in the same way as the biofilm analysis described in Section 3.4.3.

The culturable cell counts were obtained by serially diluting 1.0 ml of effluent sample in Ringer solution and spread plating on TSA plates (3.0 g/l). The plates were incubated at room temperature for up to two weeks. Effluent optical density was determined

spectrophotometrically at a wavelength of 600 nm. Finally, cell viability values, as determined by BacLight™ were correlated with culturable cell counts, while cell abundance as determined by measuring total filter area coverage was correlated with corresponding OD values.

3.5 INFLUENCE OF NUTRIENT CONDITIONS ON BIOFILM ANTIMICROBIAL SUSCEPTIBILITY

3.5.1 Experimental set-up

The flow cell microcosm set-up has been described in Section 3.4.1. Modifications to the procedure are listed below. For each nutrient condition, a set of 4 channels was used. This allowed duplicate flow channels for control and biocide treatment to assess the effect of biocide treatment on mixed-species biofilm viability and behavior.

For the first 24 h, the biofilm community in each flow channel was supplied with TSB (3.0 g/l), after which the medium was changed to one of the other nutrient conditions (see Section 3.5.2) for another 48 h. The three-day-old biofilm community was treated with a commercial biocide (a mixture of isothiazoline and gluteraldehyde). The biocide was added to the test medium at a final concentration equivalent to 90% killing efficiency in TSB (3.0 g/l) medium, and applied for 5 h at a constant rate of 5.5 ml/h. After 5 h, the flow channels were reconnected to the medium lacking biocide and the effluent collected for 30 min.

3.5.2 Nutrient conditions

The nutrient sources chosen were TSB and a minimal salt medium (MSM) supplemented with D-Glucose (Merck Laboratory Supplies (PTY) Ltd., South Africa). The minimal salt medium (Wolfaardt et al 1994b) consisted of (per 1.0 liter) Solution A: 2.00 g NaCl, 1.00 g NH_4Cl, 0.12 g $MgSO_4$ $7H_2O$, 1.0 ml trace element solution (40 mg $CuSO_4$ $5H_2O$, 500 mg H_3BO_3, 100 mg KI, 120 mg $FeCl_3$, 40 mg $MnSO_4$ H_2O, 215 mg Na_2MoO_4 $2H_2O$, 400 mg $ZnSO_4$ $7H_2O$, 1000 mg NaCl, 100 mg $CaSO_4$, 132 mg $CaCl_2$ 2 H_2O, and 10 mg Al $K(SO_4)_2$ $12H_2O$, in 1000 ml ddH_2O) and Solution B: 4.24 g Na_2HPO_4, and 2.70 g KH_2PO_4. Solutions A and B were autoclaved separately, then mixed before application. The carbon source was

added to solution A before autoclaving. The different nutrient concentrations were, 3.0, 0.3, and 0.1 g TSB /l H_2O, or MSM + 1.0, 0.1, or 0.0 g Glucose/l.

3.5.3 Biocide application

In a preliminary series of experiments the efficiency of the commercial biocide was evaluated against planktonic cells. Several different concentrations, including the recommended, and higher concentrations of the stock solution were tested. A concentration that resulted in a 90% killing of the planktonic microbial community was subsequently used in all experiments involving biocide treatment.

3.5.4 Cultivation of biofilms

Biofilm cultivation was previously described in Section 3.4.2. The biofilm community consisted initially of 7 bacterial isolates (Table 4.3.1), but some isolates were difficult to distinguish from each other on TSA plates, complicating determination of population ratios. Therefore, a mixture of 4 easily distinguishable isolates, namely CT01, CT03, CT04 and CT07, was used as inoculum for the subsequent experiments.

3.5.5 Biofilm analysis

Biofilm antimicrobial susceptibility was monitored in terms of biofilm viability and area coverage (biofilm density). Flow channels were stained with BacLight^{TM} and analysed using an epi-fluorescence microscope and image analysis (see Section 3.4.3 for detailed analysis of procedures).

3.5.6 Analysis of planktonic cells

Effluent (3.0 ml) of each flow channel was sampled before and directly after the biocide treatment. Culturable cell counts, cell viability and abundance on polycarbonate filters, as well as optical density were determined to observe the effect of the biocide on the planktonic communities. Refer to Section 3.4.4 for detailed description of the procedures.

3.5.7 Influence of nutrient conditions on biocide efficiency

To evaluate the potential effect of the growth media on biocide efficiency, the biocide was diluted in the two nutrients, at concentrations as previously described (Section 3.5.2).

The isolates (Table 4.3.1) were grown overnight in 3.0 g/l TSB and mixed into two different combinations, i.e., Mix4 (CT01, CT03, CT04 and CT07) and MixA (the seven original isolates except CT06). A 0.2 ml aliquot of each mixture was then spread in duplicate onto 3.0 g/l TSA plates. Once the plates were dry, they were divided into 7 equal parts and spotted with 0.01 ml of each of the biocide dilutions. Water without biocide was used as negative control. The efficiency of the biocide was determined by measuring the diameter of the clear zones that formed in the microbial mat.

3.6 BIOFILM AND MICROBIAL RECOVERY UNDER TWO DIFFERENT NUTRIENT CONDITIONS AFTER BIOCIDE TREATMENT

3.6.1 Experimental set-up

The same flow cell set-up was used as described in Sections 3.4.1 and 3.5.1. Three-day-old mixed-species biofilms were allowed to recover for 7 days after biocide treatment. All tests were done in duplicate.

3.6.2 Cultivation of biofilms

Biofilms were cultivated for 3 days as described in Section 3.4.2. Bacterial isolates used for the defined biofilm community were CT01, CT03, CT04 and CT07 (Table 4.3.1).

3.6.3 Nutrient conditions

Nutrient concentrations used in this experiment were 3.0 g TSB / liter and MSM + 0.1 g Glucose/liter.

3.6.4 Biofilm analysis

Six flow channels were treated with the biocide. In the case of biofilms cultivated on TSB, individual flow channels were stained with the Baclight™ stain at 0, 24, 78 and 174 h (the latter in duplicate) after biocide treatment, respectively, and analyzed as described in Section 3.4.3. For the biofilms cultivated on glucose, sampling was performed at 0, 24, 78, 126 and 174 h after biocide treatment. An extra sampling time (126 h) was added for allowing better monitoring of biofilm recovery. Two flow channels served as untreated controls; one was analyzed at time 0, and the other after 174 h.

3.6.5 Analysis of planktonic cells

Cell viability and abundance, culturable cell numbers, and population ratios were determined for the planktonic communities. Cell numbers were determined to observe the effect of the biocide on the biofilm-derived planktonic cells and the possible trends in the abundance of the different isolates over the recovery period. Terminal Restriction Fragment Length Polymorphism (T-RFLP) and fluorescent *in situ* hybridization (FISH) were used for culturable-independent analyses of population dynamics.

Effluent was collected within 1 h before and 1 h after the addition of the biocide, and then every 12 to 24 h. For T-RFLP analysis, 2.0 ml of the effluent was centrifuged at 13 000 rpm for 6 min. The cell pellet was then resuspended in 500 ml TE buffer and stored for further analysis. One to two ml of each effluent sample was washed, fixed and stored for further FISH analysis. Both samples were stored at –20 °C. Protocols for T-RFLP (Liu et al 1997) and FISH are discussed in the following (Section 3.7).

3.7 BIOFILM POPULATION DYNAMICS

3.7.1 Population ratios determined by conventional spread plating techniques

Numbers of the respective isolates present in the effluent samples were determined using spread plates (3.0 g/l TSA). Colony pigmentation or shapes were used to differentiate between isolates.

3.7.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Stored effluent samples, collected from previous experiments (Section 3.6), were thawed for genomic DNA extraction. Samples taken at day 0 (just before biocide treatment), day 1 (24 h after biocide treatment), day 3 (78 h after biocide treatment) and day 7 (174 h after biocide treatment) were chosen for T-RFLP analysis. Genomic DNA of the four isolates was extracted and analyzed for the determination of isolate specific restriction terminal fragment (T-RF's) sizes. The lengths obtained were then compared with the theoretically determined 16S rDNA terminal fragments as determined by the PC-based DNAMAN (Version 4.1) software from Lynnon Biosoft.

3.7.2.1 Extraction of bacterial genomic DNA

Several bacterial genomic DNA methods were evaluated for a protocol to extract Gram negative and Gram-positive genomic DNA to minimize extraction bias between the four isolates. The best DNA representations were obtained using the CTAB based DNA isolation method described by Ausubel et al (1993). The method was adjusted and optimised as follows:

Flow channel effluent samples (1.5 ml) were centrifuged for 6 min at 13 000 rpm (bench top centrifuge, Biofuge 13). Harvested cells were then resuspended in 500 µl TE buffer (10mM Tris, 1mM EDTA, pH 8) and stored at –15 °C. Samples were thawed and centrifuged for 10 min at 13 000 rpm and resuspended in 555 µl TE buffer. Bacterial cells were treated with 5.0 µl of lyzozyme (50 mg/ml) and incubated for 60 min at 37 °C. For cell lysis and protein degradation, 30 µl 10% SDS and 10 µl Proteinase K (20 mg/ml) were added. The solution was well mixed and incubated for 1 h at 45 °C. Thereafter, 100 µl of 5M NaCl and 80 µl CTAB/NaCl solution was added and incubated at 65 °C for 10 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added and spun for 5 min at 13 000 rpm. The top layer was transferred to a fresh tube. The DNA was then precipitated with 2 volumes of 96% ethanol and stored overnight at –15 °C. DNA was pelleted by centrifugation at 13 000 rpm and washed with 100 µl 70% ethanol. The DNA was dried at 37 °C for 5 min and resuspended in 100 µl of TE buffer. Successful DNA isolation of each effluent sample was confirmed by loading 5 µl of DNA extract on a 0.8% agarose gel for electrophoresis at 80V for 15 min.

3.7.2.2 PCR protocol

Due to varying numbers of cells in the different test samples, different concentrations of total genomic DNA were obtained. The amount of genomic DNA used for optimizing the PCR was chosen according to the approximate brightness of genomic DNA bands (representing DNA concentrations) following electrophoresis on a 0.8% agar gel. The 16S rRNA gene was amplified using a reaction mixture of 1-4 µl of genomic DNA, 0.25 µM of each primer, 25 µl of PCR Master Mix (Promega Corp.) and double distilled water for a final reaction volume of 50.0 µl. The primers used were a 5'-FAM-labeled 341F (5'-CCTACGGGAGGCAGCAG-3') and the 3'-HEX-labeled 1389R (5'-ACGGGCGGTGTGTACAAG-3'). PCR was performed in a Perkin-Elmer thermocycler (Gene Amp 2400) using an initial denaturation step of 94 °C for 1 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s. A final extension step at 72 °C for 10 min was performed after the programmed number of cycles was completed.

Two restriction enzymes, *Rsa*I and *Alu*I (Roche Molecular Biochemicals), were used for two separate restriction enzyme reactions of each test sample. Restriction digest reactions were incubated for 4 h at 37 °C, followed by an enzyme-denaturing step at 70 °C for 15 min. Restriction enzyme digests were cleaned by either ethanol precipitation (Sambrook et al 1989) or CENTRI SEP spin columns (Prinston Seperation).

3.7.2.3 T-RFLP analysis

Two microliters of the restricted PCR product was analyzed. DNA fragments were separated on an ABI Prism® 3100 Genetic Analyzer. Terminal fragments were detected by excitation of the FAM or HEX molecule attached to the 5' or 3' ends of the fragments. Fluorescence intensity of the terminal fragment lengths was analyzed by using analysis software, Genescan Analysis Version 3.7 (Applied Biosystems). A peak height threshold of 100 fluorescence units was used.

3.7.3 Fluorescent *in situ* hybridization (FISH)

3.7.3.1 Fixation methods

Two fixation methods were compared since one of the four isolates was a Gram-positive bacterium. Paraformaldehyde (4%) is generally used to fix Gram-negative bacteria, while it has been found that ethanol fixation is more successful with Gram-positive bacteria. The eubacterial probe, Eub 338 (rhodamine- 5'-GCTGCCTCCCGTAGGAGT-3') (Amann et al 1990a), was used at a concentration of 50 µM. Pure cultures were grown overnight and then harvested at 13 000 rpm for 3 min. The supernatant was discarded and the cells were washed with 1x PBS (1M NaCl, 0.2M Na_2HPO_4, 0.2M NaH_2PO_4, pH 7.2-7.4). The washed cells were spun down and then resuspended in 500 µl 1xPBS. For 4% paraformaldehyde fixation, washed and resuspended cells were stored overnight at minus 20°C after 3 volumes of 4% paraformaldehyde were added. Cells were centrifuged at 13 000 rpm for 5 min, washed in 1x PBS, centrifuged again and finally resuspended in 500 µl of 1x PBS and 1 volume of ice cold ethanol (96%). The fixed cells were stored at -20 °C for further analysis. For ethanol fixation, only one volume of ice cold 96% ethanol was added to the washed and resuspended cells and stored at -20 °C.

3.7.3.2 Hybridization on glass slides

Ten microliter of fixed cells were added per well on a glass slide and incubated at 60 °C for 20 min. The heat fixed cells were then dehydrated step-wise in 50%, 80% to 100% ethanol, each for 3 min. The slides were air-dried and the hybridization buffer [180 µl 5M NaCl, 20 µl 1M Tris, MilliQ water (total volume of 1000 µl), percentage formamide according to stringency, and 1.0 µl 10% SDS] was prepared. The fixed cells were probed with a mixture of 9.0 µl of hybridization buffer and 1.0 µl fluorescently labeled oligonucleotide probes. Humid conditions were maintained by adding hybridization buffer to a hybridization tube containing paper cloth and the probed slide was incubated for 2 h at 46 °C. During hybridization, the wash buffers (corresponding % formamide used) were prepared and pre-warmed to 48 °C in a water bath. After hybridization, the slides were carefully rinsed with pre-warmed wash buffer and then incubated in the wash buffer for 10-15 min at 48 °C. The slides were then rinsed with milliQ water, air-dried, and stored at –20 °C for later analysis.

3.7.3.3 Design and evaluation of specific a FISH probe for the bacterial isolate CT04 (*Dyadobacter* sp.)

A specific oligonucleotide probe was designed for the detection of bacterial isolate CT04 within the flow channel planktonic population. This population consisted of four different bacterial isolates. The three partially known 16S rDNA sequences of isolates CT03, CT04 and CT07 (Section 3.3.2) were aligned against the known 16S rDNA sequence of *Esherichia coli* K-12, (NCBI Accession No X80725) using PC-based software DNAMAN. Non-conserved regions were selected and base positions were estimated according to the 16S rDNA sequence of *E. coli* K-12 to evaluate the hybridization/ annealing efficiency on the folded 16S ribosomal DNA molecule (Behrens et al 2003).

The specific oligonucleotide probe sequence (5'-ACCAATCGTAGCCACC-3') was labeled at the 5' end with fluorescein. Evaluation of the probe hybridization stringency was done by testing the probe against a series of different hybridization conditions by changing the formamide concentrations in the hybridization buffer. Best working stringency hybridization conditions were achieved by using 10% formamide. Probe specificity was tested by probing it against the other members of the bacterial community in pure culture using the FISH protocol described in the previous Section 3.7.2.2.

3.7.3.4 Detection of bacterial isolate CT04 in the flow channel effluent bacterial community

Ethanol-fixed samples from the effluents collected during the biofilm regrowth experiments (Section 3.6) were analyzed for the presence of the bacterial isolate CT04 using FISH and incorporating the probe as described in Section 3.7.3.3. Samples taken at days 0, 3, 6 and 7 were spotted in duplicate onto glass sides, fixed and probed following the protocol described above. The specific probe for isolate CT04 was mixed with the hybridization buffer [180 µl 5M NaCl, 20 µl 1M Tris, 100 µl formamide (10% formamide), 700 µl MilliQ H_2O and 1.0 µl SDS] and washed with the corresponding wash buffer (4.50 ml 5M NaCl, 1.00 ml 1M Tris, 44.45 ml MilliQ H_2O) after hybridization. Probed samples were counterstained with 2.50 µg/ml DAPI fluorescent probe solution for 5 to 10 min, rinsed with MilliQ H_2O, and air-dried. Stained samples were analyzed using epi-fluorescence microcopy. Images were collected using a digital camera (COOLPIX 990, Nikon, Japan) mounted on the microscope.

CHAPTER 4:
RESULTS AND DISCUSSION

4.1 COMPARISON OF ABUNDANCE AND ANTIMICROBIAL SUSCEPTIBILITY OF BIOFILM AND PLANKTONIC MICROBIAL COMMUNITIES IN AN OPERATIONAL COOLING TOWER SYSTEM

The aim of this part of the study was to observe the microbial ecology of a water-cooling system under routine chemical treatment. Chemicals were added once a week to the system to control corrosion and microbial activity. The impact of biocide on microbial growth and viability within the water-cooling tower was the primary interest.

4.1.1 Biofilm development and antimicrobial susceptibility

4.1.1.1 Biofilm viability

After a four-day exposure to the water-cooling environment, 90% of the biofilm covering the test surfaces was viable (FIG 4.1). Biofilm viability decreased to 70% on day 7, being followed by a further decrease within the next 24 h. Viability of the 8-day-old biofilm was 10%, which indicated that the expected biocidal effect had occurred. Recovery in biofilm viability took place over the following few days. The sudden decrease in biofilm viability on days 11 and 18 (FIG 4.1) was unexpected, notably because the maintenance personnel confirmed that no deviations would be made to the treatment schedule. The unexpected decrease in biofilm viability could be due to drastic temperature changes or other unknown environmental or operational factors. On days 14 and 15, no drastic change in the biofilm viability was observed although a chemical had been added, as indicated by a color change in cooling tower water. The drastic increase from 20 % to 65% viability on the 18th to the 21st day might be explained by high temperatures and humid conditions experienced during that time. The fluctuating pattern of the last recording days could be attributed to weather changes, the analysis of an unrepresentative number of microscope images, or interference of EPS with accurate thresholding of images.

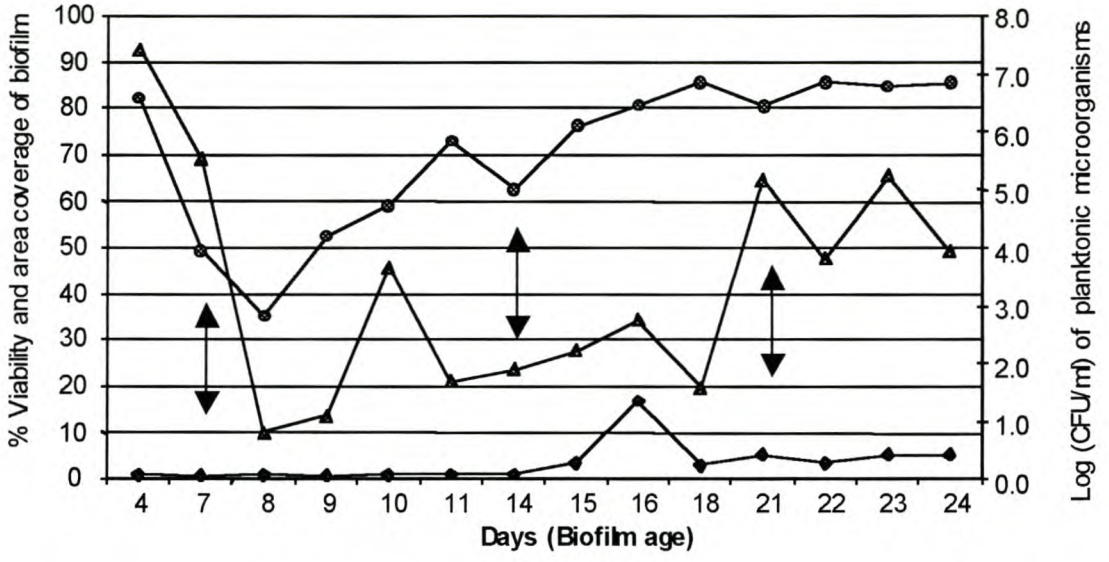


FIG 4.1 Biofilm and planktonic cell behavior within an operational cooling tower system in terms of percentage biofilm viability (triangle) and area coverage (diamond), as well as culturable planktonic microorganisms [log (CFU/ml)] (circle). The system was exposed to a scheduled chemical treatment, occurring every seven days (arrows).

Results obtained by image analysis agreed with visual microscopic observations. For instance, microscopic observations showed active cell division of microbial cells in 4-day-old biofilms. A drastic change in biofilm viability was observed within a day after the biocide treatment (day 7). A few small areas within the biofilm contained viable cells. These cells were predominantly positioned beneath the non-viable cells. This heterogeneity in the distribution of viable cells was observed throughout the observation period. Similar biofilm heterogeneity has been described by other researchers (Lawrence et al 1996; Korber et al 1997).

The chemical used in the second week showed no effect on the biofilm, as a large number of viable cells were present in the biofilm one day after the treatment on day 14. Interestingly, over the next few days, the number of viable cells and microcolonies slowly decreased. Four days after the second chemical treatment, the biofilm appeared less dense and some areas of the slide were void of cells.

The 21-day-old biofilm differed markedly from the biofilms observed at earlier stages. Viable cells dominated and were positioned above the non-viable cells. Numerous microcolonies could be observed and the biofilm increased in density. No drastic changes in biofilm viability were observed after day 21. Visual observations supported the data obtained by image analysis that biocide treatment influenced biofilm structure and abundance, in addition to the impact on the biofilm viability, as described above.

4.1.1.2 Biofilm area coverage

No major changes in biofilm area coverage from day 4 to day 14 were detected with image analysis, while a slight increasing trend could be observed after day 14. The 11-day-old biofilm contained three-dimensional (tower-like) microcolonies. The number of colonies increased over the next few days containing large spherical cells, possibly yeasts. The drastic change in area coverage on days 16 and 17 (FIG 4.1) could be due to non-representative image capturing (non-random selection for microcolonies) or deposits.

Regular direct microscopic observations provided insight into initial biofilm development under these *in situ* conditions. For instance, the surface of the glass slide was sparsely covered with rod shaped bacteria after 24 h. Over the next days the biofilm area coverage increased slowly, forming the first microcolonies after the third day and cell distribution changed from even to uneven due to microcolony formation. Biofilm coverage did not decrease in response to biocide treatment on day 7. The same was initially observed after the second treatment on day 14, but within two to four days the biofilm appeared less dense with fewer microcolonies. Computer analysis of these images showed that there was indeed an increase after day 14, and only after day 21 was there a positive correlations between the observations made by direct microscopy and the results obtained by image analysis. Overall, image analysis showed that biocide treatment had no marked influence on biofilm area coverage. Direct microscopic observations, taking subjectivity in account, appeared to be more sensitive in terms of detecting changes in biofilm abundance and structure that were not detected with the image analysis approach. Possible reasons could be that the image analysis approach is less sensitive or less representative due to a variety of factors e.g., too few images analyzed. On average, biofilm area coverage on glass slide was less than 10%. This may also have contributed to the apparent lack of sensitivity in terms of biofilm area coverage.

Despite the degree of inconsistency between direct observations and image analysis, it is evident that the chemical treatment did not remove biofilms (MacDonald et al 2000). It is likely a general phenomenon that inefficient biofilm removal leads to conditions where the remaining biofilms provide a protective environment to viable cells, which could subsequently act as a cell reservoir for rapid regrowth and reattachment. This may lead to a fast recovery of microbial growth, requiring increased frequency in biocide application, or may even lead to the selection of biocide resistant microbial populations (Cloete et al 1998; Flemming 2002).

4.1.2 Behavior of planktonic cells in cooling tower water

Biocide treatment on day 7 caused a significant decrease in planktonic cells (FIG 4.1) and it took more than a week to recover to their initial numbers. The second chemical treatment on day 14 had a smaller impact, and cell numbers recovered faster after the second treatment and remained relatively constant over the rest of the testing period. No change in planktonic numbers was observed after day 21, which could be due to reaching numbers close to the carrying capacity of the cooling water, as well as the lack of biocide treatment. The increase in biofilm area coverage showed a positive correlation with the increase in planktonic numbers.

4.1.3 Initial cell attachment and biofilm development after biocide treatment

One day after the first biocide treatment (day 8), the 24-h-slide (a new slide that had been added on the previous day) showed the attachment of only a few non-viable cells. Similar observations were made on test slides introduced daily into the system for the next 4 days. This suggests that the biocide had a long-lasting effect on initial cell attachment either due to decreased planktonic cell numbers or by creating unfavorable conditions preventing cell attachment. The latter reason may be a more likely explanation as the culturable cell count increased over that period of time. The second chemical treatment (day 14) appeared to have no effect on initial cell attachment. The slide that was exposed for 24 h (added on day 14 and sampled on day 15) showed notable cell attachment with the initiation of microcolony

formation. Four days after biocide treatment, a slide exposed to the cooling tower water for 48 h showed a biofilm consisting of evenly distributed viable cells.

As stated earlier, the objective of this part of the study was to observe biofilm behavior in a water system subjected to a routine chemical treatment. This was performed as a "blind test" without prior knowledge of the nature of the chemicals that were applied. After conclusion of the tests, it was established that the first chemical (day 7) was indeed a biocide/dispersant combination, and the second (day 14) a corrosion controlling agent. This information explains the difference in initial biofilm formation after each chemical treatment. Dispersants condition surfaces in such a way that cell attachment is hindered or prevented (Eginton et al 1998; Azeredo et al 2003).

4.1.4 Conclusion

Several key observations were made. Firstly, heterogeneity in biofilm area coverage and viability was detected, and uneven distributed areas of either, dominant viable or dead cells were found, especially in treated and older biofilms. Secondly, uneven distribution of microcolonies in the initial biofilm developmental stage, and the formation of tower like colonies in latter biofilm developmental stage made accurate representative image analysis difficult (selection bias). Thirdly, a decrease in biofilm viability was not always accompanied by a decrease in planktonic cell numbers (day 11, day 22). This stands in contradiction to observations reported in literature (LeChevallier et al 1988; Gilbert et al 1990), claiming that planktonic cells are more susceptible to antimicrobial treatment than biofilm cells.

The questions that arose included whether biofilm heterogeneity was affecting image analysis, and how to statistically capture the heterogeneity of a biofilm sample for accurate image analysis.

4.2 INFLUENCE OF HETEROGENEITY ON REQUIRED SAMPLE SIZE

Heterogeneity in biofilm viability and area coverage on glass slide surfaces were observed in the previous set of experiments (Section 4.1), which led to the question of sample size needed for accurate image analysis. The aim of this study was therefore to determine the influence of heterogeneity on the sample size needed for statistical representation of average biofilm and planktonic cell viability and area coverage when using BacLight™, a fluorescent bacterial viability stain.

4.2.1 Biofilm heterogeneity and sample size

Heterogeneity of the biofilms on control (no biocide treatment) and replicate biocide-treated glass slides were determined by applying image analyses in terms of percentage area coverage and the percentage biofilm viability. The effect of biofilm age was taken into account by analyzing 4-, 7- and 14-day-old biofilms.

4.2.1.1 Biofilm area coverage

Biofilm area coverage increased over time from 1.5% to 5%, with no significant difference between treated and untreated biofilms over the testing period of 14 days. As expected, average biofilm area coverage did not provide an indication of the heterogeneity in area coverage (Appendix: FIG 4.2.1.1, FIG 4.2.1.2, FIG 4.2.1.3) observed when 50 - 70 images of a biofilm were analyzed. Patterns in heterogeneity differed between the treated and untreated biofilms and within each biofilm of all sampling days.

As expected, the greater the standard deviation (heterogeneity) between the samples, the greater was the sample size required for accurate and representative image analysis. The number of images for the biofilm analysis often varied drastically between the two statistical equations (Section 3.2). Based on equation [1], the number of required images calculated for the 4-day-old biofilms (both biocide treated and untreated) ranged between 70 and 220 images, while according to equation [2] only 40 to 70 images were needed. An even greater image sample size was needed to analyze the 7-day-old biofilms. Interestingly, fewer images were needed to assess the 14-day-old biofilms. There was also significant variety within treatments. For instance, in the case of biocide-treated biofilms, the number of images needed ranged between as 46 and 60, respectively, for a 4-day-old biofilm (Appendix: FIG

4.2.1). This could be an indication of the difference in heterogeneity, which is not revealed by only determining the average area coverage.

Equation [2] was chosen for determining an average number of images needed for accurate biofilm analysis. This number could only be determined after analyses were done, for image processing was needed before statistical evaluation could be carried out. Thus, based on results discussed above, it was decided to analyze on average 60 images for glass slides, and 30 – 40 images for flow cell channels (Appendix: FIG 4.2.2). Calculations of the required sample size for analyzing flow channel images showed that fewer fields were needed for statistical representation (data not shown).

4.2.1.2 Biofilm viability

Average biofilm viability for the untreated biofilms at days 4, 7 and 14 was 45%, 60% and 33%, respectively. For the treated slides, these values were 39%, 27% and 39%, respectively. In general, the viability differed from one image to the next, at times ranging from 0% to 100% within a biofilm analyzed. Heterogeneity appeared to be independent of biocide treatment (Appendix: FIG 4.2.2.1, FIG 4.2.2.2, FIG 4.2.2.3), for the viability of the untreated biofilm varied as much as that of the treated biofilm. The low viability of the control biofilms may be due to chemical treatment of the cooling tower water prior the sampling of the biofilm-covered slides.

It was found that the number of images required for biofilm viability analysis did not differ notably from the numbers calculated for biofilm area coverage analysis. Similarly, treated and control biofilms also required comparable numbers of images for analysis.

4.2.2 Assessment of sample size for planktonic cells on filters

The number of images needed for representative cell viability and relative abundance (filter area coverage by total biomass) ranged from 20 - 30 images per filter analyzed. No difference in number of images for viability and relative abundance was observed, suggesting that the cells were evenly distributed on the filters.

4.3 ISOLATION AND CHARACTERISATION OF BACTERIAL ISOLATES FROM COOLING TOWER WATER

A fact that became apparent during the study of microbial behavior in the cooling tower is that an accurate comparison between biofilm and planktonic behavior would not be possible, due to a number of variables involved (e.g., temperature, biocide addition and nutrient conditions in the open system). It was therefore decided to isolate a number of bacterial strains from the cooling water for the establishment of a defined mixed-species community, which could be studied in a controlled system.

Several bacterial species, isolated from the cooling tower water, were selected to study biofilm behavior and population dynamics when exposed to biocide and different nutrient conditions. Acknowledging the shortcomings of culture-dependent techniques in microbial ecological studies (Amann et al 1995), the results of the culture-dependent techniques were compared with two culture-independent techniques, namely T-RFLP and FISH (Section 4.7).

4.3.1 Defined microbial community

Seven bacterial isolates were selected based on their colony shape and pigmentation. The differences in phenotype between the bacterial isolates are summarized in Table 4.3.1.

4.3.2 Identification of bacterial isolates by 16S rDNA sequencing

Partial 16S rDNA sequences of four selected isolates were obtained. Isolates CT03 and CT07 were identified as two different Pseudomonads. The closest *Pseudomonas* sp. determined by BLAST analyses of the 16S rDNA sequences were *Pseudomonas veronii* [99% nucleotide identity (1404/1405 nucleotides), NCBI accession no. AY081814] for isolate CT03, and *Pseudomonas migulae* [99% nucleotide identity (900/905 nucleotides), NCBI accession no. AFO74383] for bacterial isolate CT07. Isolate CT04 was identified as a *Dyadobacter* sp. [98% nucleotide identity (1350/1375), NCBI accession no. AF137029], while no sequence could be obtained to identify CT01.

Table 4.3.1 Characteristics of bacterial isolates

Isolate code	Colony color	Colony shape	Cell morphology	Gram stain	Isolate name
CT01	White	Small, round	rod	Positive	Unidentified
CT02	Bright yellow	Small, round	coccoid	Positive	Unidentified
CT03*	Cream	Irregular, curled	rod	Negative	*Pseudomonas* sp.
CT04	Orange/yellow	Round	rod	Negative	*Dyadobacter* sp.
CT05*	Transparent	Irregular, floral shaped	rod	Negative	Unidentified
CT06	Dark pink	Round	rod	Negative	Unidentified
CT07	Transparent	Ring shaped	rod	Negative	*Pseudomonas* sp.

*Isolated from biofilm growing in a winery cooling system

4.3.3 Specific growth rates of bacterial isolates in batch culture

Growth, and the maximum specific growth rates (μ_{max}) of batch-culture grown isolates CT01, CT03, CT04 and CT07 were determined (Table 4.3.2). Different nutrient conditions, 3.0 g/l TSB, MSM + 0.1 g/l Glucose and MSM + 1.0 g/l Glucose were tested. Lower rates were observed when the MSM + Glucose was used as growth medium for isolates CT03, CT04 and CT07, while isolate CT01 did not grow on this medium.

4.3.4 Biofilm formation rate (BFR)

For isolates cultured in 3.0 g/l TSB, the biofilm formation rate was generally lower than their μ_{max} when grown in batch cultures (Table 4.3.2). When all four isolates were grown together in a biofilm, the biofilm formation rate was slightly greater (BFR = 0.240 h^{-1}) than the fastest biofilm growing isolate (BFR = 0.216 h^{-1}). Isolates CT01, CT03 and CT07 formed biofilms within a few hours, while CT04 responded much slower (FIG 4.3.4.1). MSM + Glucose grown biofilms had a slightly different growth pattern and lower BFR values (Appendix: FIG 4.3.4.2). BFR values of mixed-species biofilms differed between the different nutrient conditions, where growth rates in TSB-grown biofilm were faster and biofilm growth thicker (higher unit values) compared to MSM + Glucose grown biofilms (Appendix: FIG 4.3.4.3).

In the case of biofilms cultured in MSM + 0.1 g/l Glucose, the BFR of bacterial isolates grown together was 0.18 h^{-1}, which was similar to the BFR of bacterial isolate CT07 (0.17 h^{-1}).

Table 4.3.2 Comparison of the maximum specific growth rates (μ_{max}) and biofilm formation rates (BFR) of bacterial isolates grown in batch and biofilm cultures, respectively.

Nutrient condition	Bacterial isolates:	CT01	CT03	CT04	CT07
0.1 g/l Glucose + MSM	Batch culture μ_{max} (h^{-1})	0.000	0.480	0.096	0.310
0.1 g/l Glucose +MSM	Biofilm culture BFR (h^{-1})	0.050	0.120	0.060	0.170
1.0 g/l Glucose +MSM	Batch culture μ_{max} (h^{-1})	0.000	0.409	0.100	0.270
3.0 g/l TSB	Batch culture μ_{max} (h^{-1})	0.250	0.570	0.330	0.520
3.0 g/l TSB	Biofilm culture BFR (h^{-1})	0.190	0.216	0.128	0.203

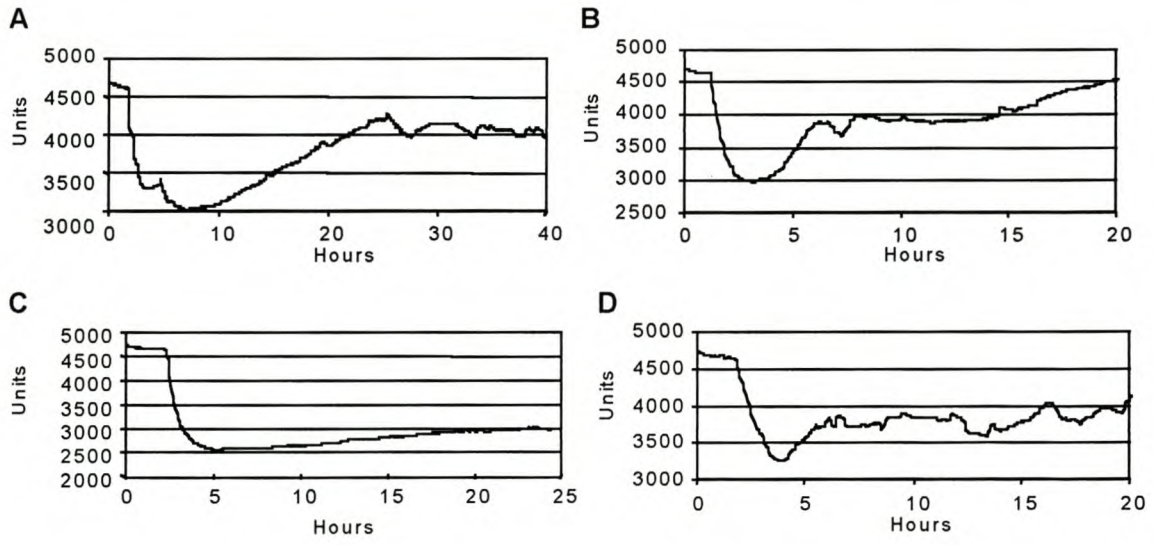


FIG 4.3.4.1 Biofilm formation curves of bacterial isolates, CT01 (A), CT03 (B), CT04 (C) and CT07 (D), grown under continuous flow conditions within an adapted flow cell system supplied with 3.0 g/l TSB. Biofilm growth was monitored with an Optical Large Area Photometer (OLAPH).

4.3.5 Interactions between bacterial isolates

No inhibition between the four isolates was observed (FIG 4.3.5). This result was expected, for the selection criteria of isolates included individual detection and identification of each isolate on TSA plates when co-cultured in a mixed-species community.

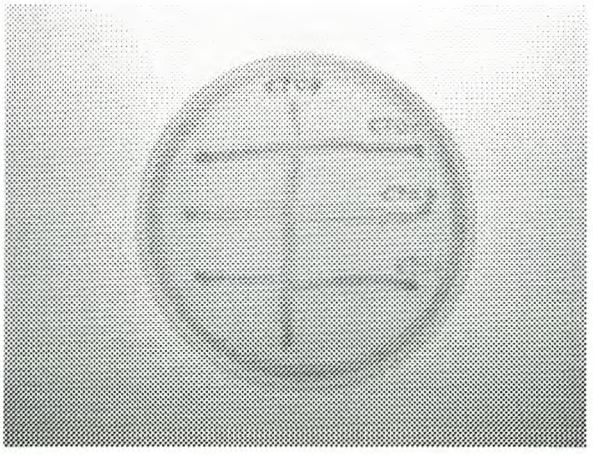


FIG 4.3.5 No inhibition was observed when the four bacterial isolates, CT01, CT03, CT04 and CT07 were cross-streaked.

4.4 DEVELOPMENT AND BEHAVIOR OF A DEFINED MIXED SPECIES BIOFILM COMMUNITY

The aim of this part of the study was to observe biofilm formation and behavior in flow cells over time, as well as to study the associated viability and abundance of planktonic microbial populations obtained from the flow cell effluent. The dominance and survival of selected bacterial isolates in the community was of further interest.

4.4.1 Biofilm analysis

Biofilm viability remained stable between flow channels over the period of three weeks (FIG 4.4.1.1). Highest viability values were reached between days one and three. Thereafter, the

viability remained stable for the duration of the experiment (21 days). Similarly, biofilm area coverage increased slowly over the first 3 days and maintained at maximum values of 25% - 30% over the rest of the experiment (FIG 4.4.1.1). The sides of the flow channel were often more densely populated than in the middle of the channel. It was also observed that the older the biofilm was, the greater was the heterogeneity in biofilm area coverage. These included areas consisting of single cell layers and parts of thick three-dimensional biofilm structures that were too dense for accurate two-dimensional image analysis.

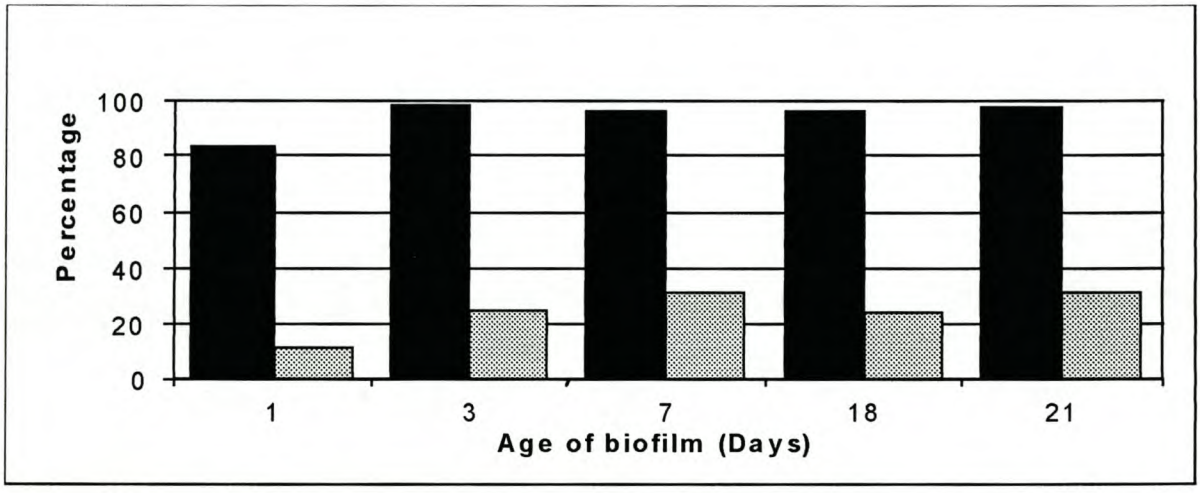


FIG 4.4.1.1 Viability (black) and area coverage (gray) of a defined mixed-species biofilm cultivated in a flow cell system over a 21-day period. Based on the reproducibility of biofilms in different channels, it was possible to use separate flow channels for the analysis of biofilms over time.

Biofilm viability was not affected by the periods of no-flow while the flow cells were disconnected from the system for microscopy analyses. This phenomenon was also observed in similar experiments carried out at a later stage of the study (Section 4.6.1).

4.4.2 Analyses of the planktonic community

4.4.2.1 Culturable cell numbers

Each flow channel was inoculated with approximately 1.15 x 10^8 CFU/ml on day 0. Twenty-four hours later, the cell count in the effluent doubled to 2.67 x 10^8 CFU/ml (FIG 4.4.2.1).

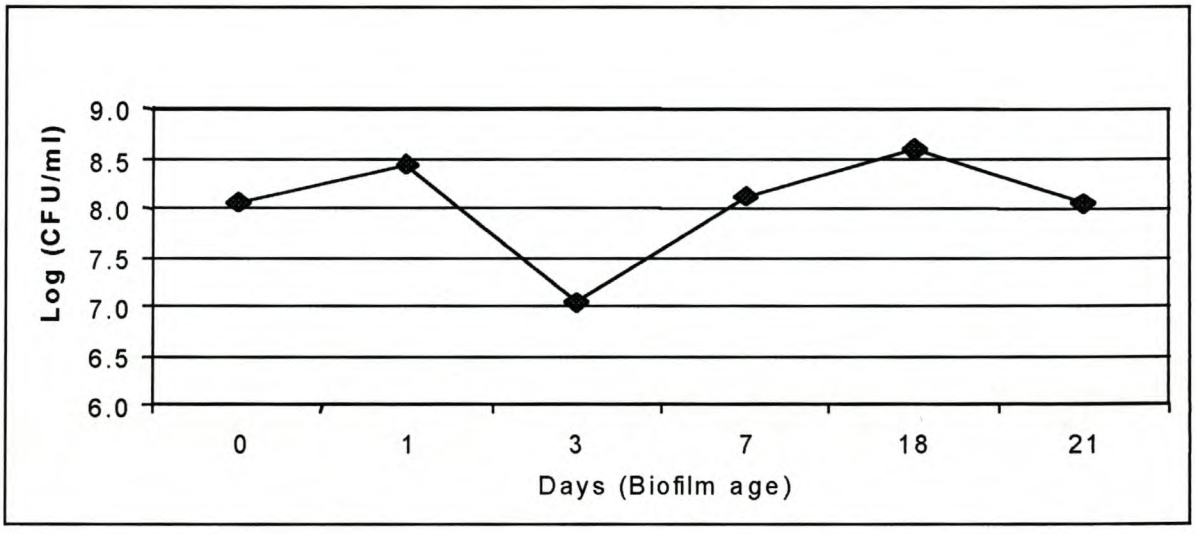


FIG 4.4.2.1 Culturable cell counts (CFU/ml) within flow channel effluent over time. The data shows that despite dilution rates exceeding μ_{max}, wash-out of the planktonic phase did not occur.

The dilution rate within the system was much higher than the μ_{max} of the fastest growing isolate. Therefore, planktonic growth could not have contributed notably to this increase in planktonic cell numbers. This has led to the question whether biofilms, even as young as 24 h, could have such an impact on their associated planktonic cell numbers. Sauer et al (2002) defined that in a steady state biofilm, the growth of biofilm is balanced by detachment. In other words, a biofilm reaches a steady state when the effluent cell counts, resulting from detached biofilm cells, remain constant. From the results above it can therefore be assumed that the biofilm community had stabilized, expressing all stages of a mature biofilm, between day 1 and day 3, and for the remainder of the experimental period. Chandy and Angles

(2001) showed that biofilms contribute to cell numbers in the aqueous phase. The different stages of biofilm development and maintenance classified in the literature are initial attachment (reversible), irreversible attachment, maturation and structure development and finally release and detachment of biofilm cells returning to planktonic growth states (Sauer et al 2002). The results of this study, together with the observations by these authors suggest that biofilms contribute to a large degree to the planktonic cells under flowing conditions that exceed specific growth rates of the community members found in the biofilms.

4.4.2.2 Planktonic cell viability and relative abundance

Planktonic cell viability, relative abundance (filter area covered by total cell biomass) and optical density of collected effluent were determined. Effluent from all flow channels was collected to compare planktonic populations derived from similar aged biofilms from different flow channels. Differences between effluent samples were observed in cell viability, relative abundance (FIG 4.4.2.2) and optical density (data not shown).

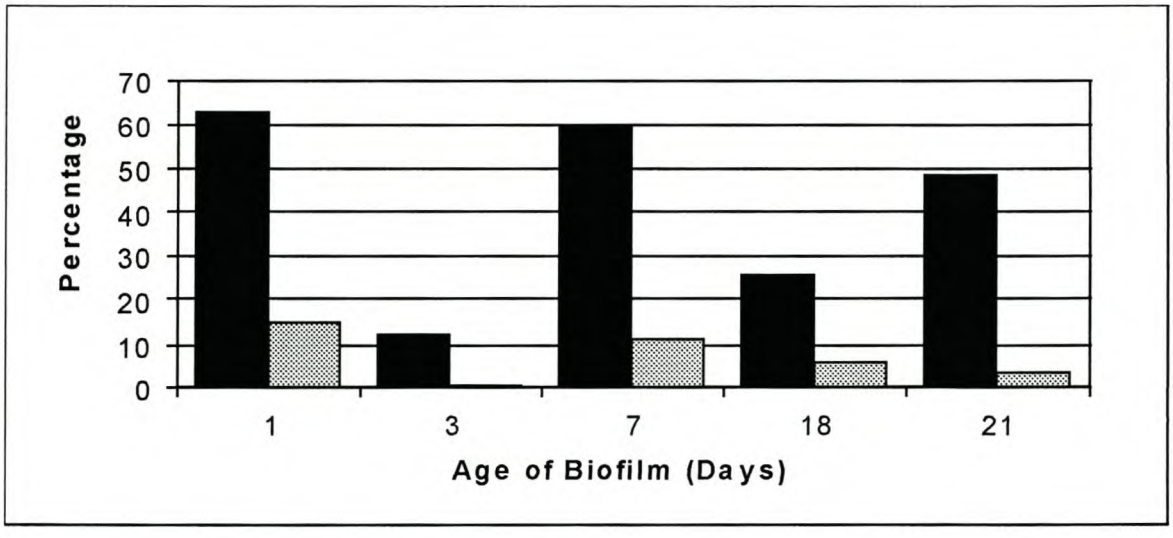


FIG 4.4.2.2 Cell viability (black) and relative abundance (gray) in flow channel effluent over time. Effluent cell viability and relative abundance were determined by staining 1.0 ml flow channel effluent with fluorescent viability stain (BacLight^{TM}) and the cells immobilized on polycarbonate filters.

Differences in optical density could have been caused by the presence of other particles such EPS, while the variation between cell viability and abundance of effluent samples could have been due to differences in shear stress and active detachment of cells.

4.4.2.3 Population dynamics

Variation in bacterial composition of flow channel effluent over time was observed (FIG 4.4.2.3). Only three isolates (CT01, CT03 and CT05) were detected on all days, while isolates CT04 and CT06 were only detected on day 3 and CT02 was absent on day 1 and 18. Despite being absent on day 1, bacterial isolate CT07 was one of the dominant isolates, and its initial absence was likely due to its slow early-stage establishment in the biofilm. Cell numbers between the different isolates ranged from 10^4 to 10^8 CFU/ml, thus the presence of isolates with low cell numbers could have been masked by the more dominant isolates.

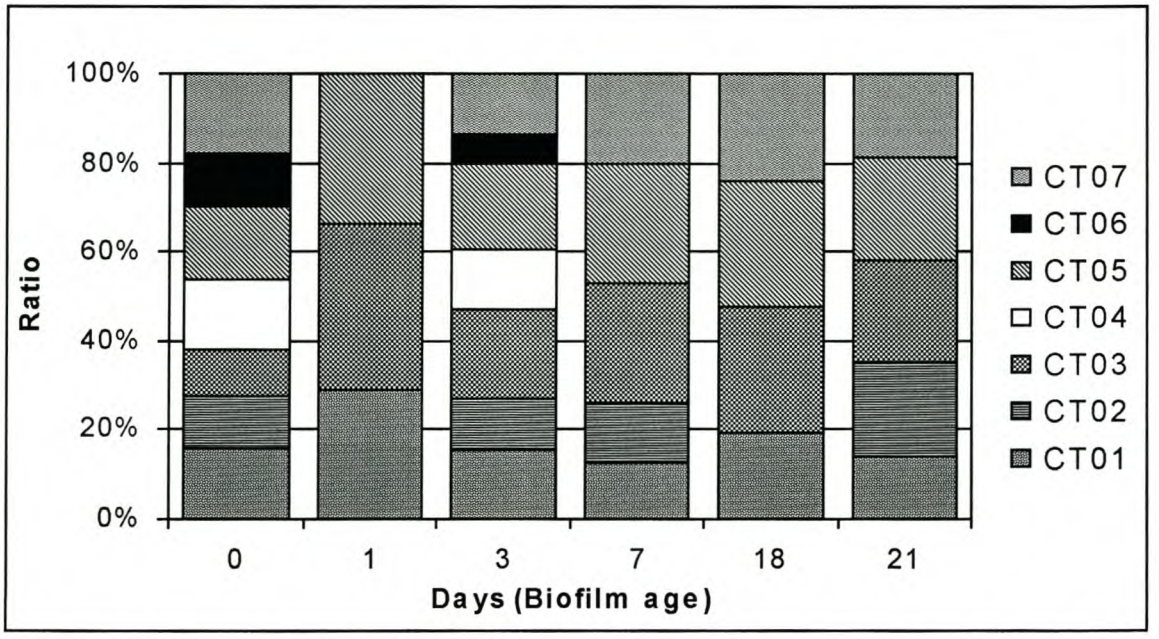


FIG 4.4.2.3 Bacterial population dynamics, in terms of percentage ratio of seven bacterial species over time. The initial bacterial population ratios (Day 0) were similar, but changed over time by the loss of isolates. Dominant populations appeared to be CT05 (unidentified), CT03 and CT07 *(Pseudomonas sp.)*, as well as CT01 (unidentified).

4.4.2.4 Correlation between culturable cell counts and cell viability, as well as relative cell abundance and optical density

No correlation (R^2=0.332) was found between culturable cell counts and associated viable cells (determined by viability staining) in the same flow cell effluent. This could be due to the phenomenon of viable but non-culturable cells (Amann et al 1995; Rockabrand et al 1999). It has been observed that not all viable cells are detected by conventional culture techniques and are therefore often overlooked. In contrast to the weak correlation between culturable cell counts and associated viable cells, optical density of effluent showed a much stronger positive correlation with associated relative cell abundance (filter area covered by cell biomass) from the same flow channel (correlation coefficients ranging between flow channels from R^2 = 0.784 to R^2 = 0.884). This observation shows that the data obtained by image analysis was reliable. Interestingly, a weak correlation in cell viability (R^2 = 0.381) was observed between planktonic and biofilm communities, and between planktonic and biofilm cell abundance (R^2 = 0.280). This lack of correlation suggests that microbial activity/viability in effluent can not be used as direct indication of biofilm activity/viability.

4.4.3 Conclusion

These observations provided insight into the behavior of biofilm and planktonic cells within a small-scale microcosm. Stable biofilm communities, in terms of constant planktonic cell counts, biofilm viability and area coverage, were observed from the third day after inoculation of the flow channels. The difference in planktonic cell viability and abundance, between the different flow channels in this study indicate that care should be taken when using only one or two flow channels for interpretation of planktonic cell activity and behavior. Culturable cell counts of effluent samples appear not to be a reliable indication of planktonic viability, while planktonic viability does not directly reflect biofilm viability. Two-dimensional image analyses using epi-fluorescence microscopy decreased in accuracy with increasing biofilm thickness.

4.5 INFLUENCE OF NUTRIENT CONDITIONS ON BIOFILM ANTIMICROBIAL SUSCEPTIBILITY

It is now generally accepted that biofilm populations are more resistant towards antimicrobials than suspended or planktonic microbial populations (LeChevallier et al 1988; Nichols 1989; Allison et al 2000; Lewis 2001). Biofilm complexity complicates the formulation of defined action-reaction behavior and leads to many answers to one question (Allison et al 2000). This is clearly seen in the effect of an environmental factor like nutrient source availability. The nature, the concentration and availability of nutrient and carbon sources in general have a profound influence on microbial behavior from biochemical reactions to growth, reproduction, and the activation of survival mechanisms. It has been suggested that nutrients influence biofilm composition and development, architectural and structural heterogeneity, microbial physicochemistry, and species diversity (Wolfaardt et al 1994a; Moller et al 1997), which may in turn impact the antimicrobial susceptibility of biofilms when exposed to antimicrobials.

4.5.1 Biofilm analysis

The susceptibility profiles of the biofilms that developed with glucose as sole carbon source were different from those exposed to a complex nutrient source (TSB) (FIG 4.5.1.1). The viability of the biofilms grown in 3.0 g/l TSB and 0.3 g/l TSB decreased from 80% to less than 10% after a five-hour biocide treatment. In contrast, when biofilms were grown with glucose as carbon source, there was no difference in viability between biocide-treated and untreated biofilms (FIG 4.5.1.1). Interestingly, the biofilms grown in 0.03 g/l TSB displayed the same sensitivity profile as the biofilms grown with glucose. At this low TSB concentration, microbial cells may have entered starvation and subsequently had a low growth rate, which has been related to antimicrobial resistance (Brown et al 1988; Evans et al 1991). Another factor adding to a less susceptible nature could be that starvation conditions induce changes in cell membrane or envelope properties (McDonnell and Russell 1999), which could decrease the detrimental impact of the biocide. Biofilm area coverage (FIG 4.5.1.2) was not impacted as drastically as biofilm viability by the different nutrient conditions.

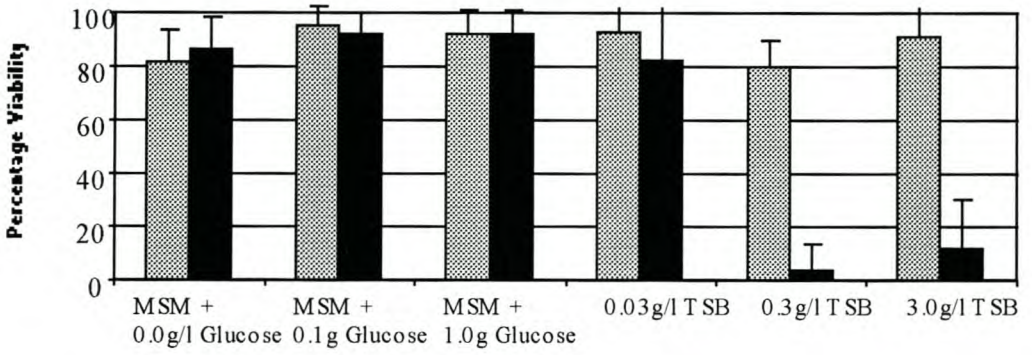


FIG 4.5.1.1 Biofilm viability of a 3-day old biofilm, grown in different nutrient conditions (MSM supplemented with glucose and TSB), was sampled 1 h before (gray) and within 1 h after (black) biocide treatment.

Antimicrobial susceptibility of the biofilms increased when grown under rich or more favorable nutrient conditions, such as TSB. The poorer the nutrient conditions became, the greater was the resistance of the biofilm community towards the biocide. The absence of peptones and other essential components in the minimal medium may have contributed towards the higher biocide resistance of biofilm and planktonic cells. Nutrient sources and conditions have been observed to influence the growth rate and growth phase of microorganisms (McLoed and Spector 1996) as well as the enzyme activity levels, EPS production (Dewanti and Wong 1995; Looitjesteijn et al 1999) and cell membrane composition (McDonnell and Russell 1999). Unfavorable nutrient conditions generally promote stationary growth phase and a decreased growth rate. Under these conditions, increased antimicrobial resistance has already been observed (Brown et al 1988; Evans et al 1991; Foley et al 1999; Xu et al 2000).

4.5.2 Analysis of planktonic community

Staining the effluent from each flow channel and subsequent immobilization of cells onto filters provided an indication of the viability (FIG 4.5.2.1) and the relative abundance (FIG 4.5.2.2) of planktonic cells. This was done due to the absence of culturable cells, on nutrient agar plates, in biocide-treated effluent.

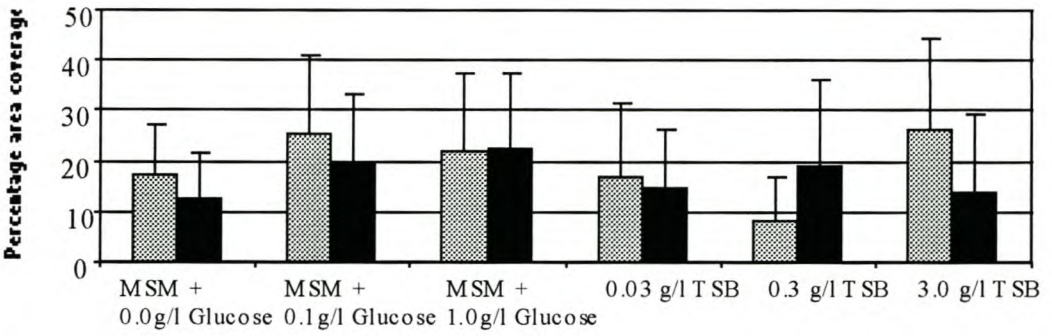


FIG 4.5.1.2 Biofilm area coverage of a 3-day-old biofilm, grown in different nutrient conditions (MSM + Glucose and TSB), was assessed 1 h before (gray) and within 1 h after (black) biocide treatment.

4.5.2.1 Microbial susceptibility (cell viability)

The viability of the planktonic populations (FIG 4.5.2.1) from the untreated flow channels ranged between 70%-90%, except for those that were supplied with MSM + 0.0 g/l Glucose (20% viable) and 3.0 g/l TSB (40% viable). Within 1 h after the biocide treatment was terminated, the viability of the planktonic populations in biocide-treated flow channels, supplied with TSB had decreased to below 10%, while the viability did not decrease below 50% in the flow channels with MSM + Glucose. Spoering and Lewis (2001) observed that slow growing or dormant planktonic cells had a higher antimicrobial resistance than fast growing cells. Interestingly, the nutrient concentration had no influence on the level of biocide sensitivity of the planktonic population in the present study, while the nutrient type impacted microbial viability. In the case of the untreated effluent population, the nutrient concentration had an influence on their viability, especially in the cases where glucose was provided as nutrient.

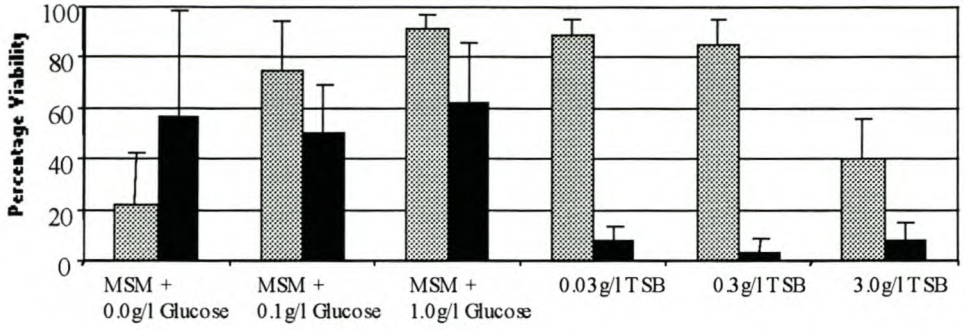


FIG 4.5.2.1 Microbial viability of the planktonic community within the effluent of flow channels containing a 3-day-old biofilm grown under different nutrient conditions (MSM supplemented with glucose and TSB) were analyzed 1 h before (gray) and within 1 h after biocide treatment (black).

4.5.2.2 Planktonic cell abundance

Under both nutrient conditions, the planktonic cell numbers increased with the increases in nutrient concentration (FIG 4.5.2.2). The relative cell abundance of the biocide-treated TSB effluent was similar to the untreated effluent, and increased slightly (10% to 20%) when nutrient concentrations increased. In contrast, very low cell abundance (2% to 4%) was observed in the biocide-treated MSM + Glucose effluent.

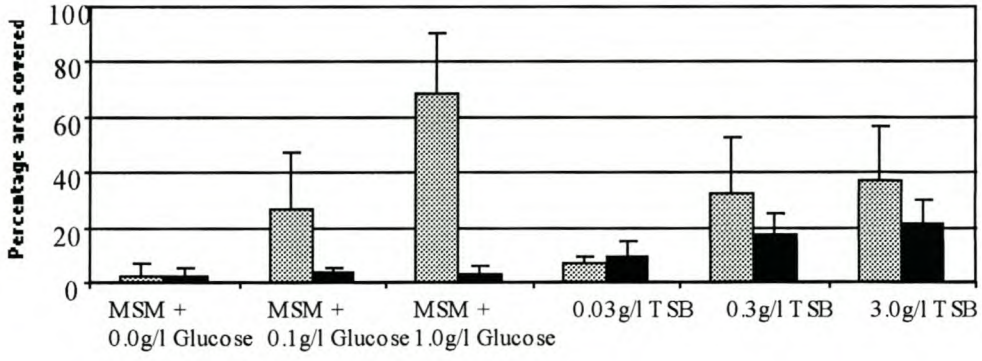


FIG 4.5.2.2 Relative cell abundance in effluent (filter area covered with cell biomass) associated with a 3-day old biofilm grown under different nutrient conditions MSM) supplemented with glucose and TSB), sampled 1 h before (gray) and within 1 h after biocide treatment (black).

Nutrient type had an impact on the degree of planktonic cell abundance, which suggests that the biocide and nutrient condition had an inhibiting influence on the shedding or detachment of cells from the biofilm. The equal abundance of the treated and untreated planktonic populations found under the low nutrient conditions of 0.03 g/l TSB and MSM + 0.0 g/l Glucose, could be an indication of the minimal amount of planktonic cells maintained (shedding or detachment from biofilm) in the effluent associated with a biofilm, irrespective of the conditions (FIG 4.5.2.2). Even though the abundance in treated and untreated effluent was the same, planktonic cell viability differed (FIG 4.4.2.1). The increase in susceptibility of the planktonic population under 0.03 g/l TSB may have been due to the biocide treatment. The increase in resistance of the treated planktonic population under MSM + 0.0 g/l Glucose, suggests that the biocide somehow triggered the preferential release of viable cells.

Since the dilution rate (D =20.75 h^{-1}) in the flow channels was 34 times higher than the fastest specific growth rate ($\mu_{max} \pm 0.6\ h^{-1}$) of the bacterial isolates (Table 4.3.2), it was evident that the majority of the planktonic cells originated from the biofilm. From this, it was proposed that the nutrient conditions in combination with biocide treatment influenced not only antimicrobial susceptibility, but also the shedding, detachment or replication of biofilm cells. This may have far-reaching implications for industrial fluid-surface based systems where the degree of impact of biofilms can be directly correlated with an increase in the surface-to-volume ratio of the system. Biofilm growth on such surfaces could act as a constant source of planktonic cells, which could be of benefit or predicament.

4.5.3 Influence of nutrient conditions on biocide efficiency

Agar spot test showed that biocide dilution in different nutrient sources and concentrations had no effect on biocide efficiency (Data not shown), confirming that the observed antimicrobial resistance (for MSM + Glucose) was not the result of the biocide inactivation by the nutrient.

4.5.4 Conclusion

Differences in the antimicrobial sensitivity (viability) profiles, before and after biocide treatment, were influenced by the different nutrient sources and concentrations. A surprisingly low antimicrobial sensitivity towards the commercial biocide was observed in biofilm populations grown when glucose was provided as sole carbon source. The same was observed for the effluent populations under these conditions, although the planktonic population was slightly more sensitive. Nutrient type and concentration, as well as biocide treatment, influenced the relative abundance of the planktonic microbial effluent population. Biocide treatment appeared to have an inhibiting influence on the detachment of biofilm cells after biocide treatment.

The next questions to be addressed were the re-establishment of biofilm viability and the regrowth rate of the mixed-species biofilm, including possible population shifts caused by biocide treatment.

4.6 RECOVERY OF BIOFILM AND PLANKTONIC CELLS UNDER DIFFERENT NUTRIENT CONDITIONS AFTER BIOCIDE TREATMENT

Following the difference in antimicrobial susceptibility of biofilms observed in the previous section, it was of interest to study the recovery and regrowth of biofilm and planktonic communities under the two different nutrient conditions after biocide treatment.

4.6.1 Biofilm analysis

The standard deviation in biofilm viability within a flow channel (FIG 4.6.A) was generally greater than between the duplicate experiments (FIG 4.6.B). Similarly, high standard deviations in biofilm area coverage were observed within each flow channel (FIG 4.6.1.A), but were found to be low between flow channels (FIG 4.6.1.B).

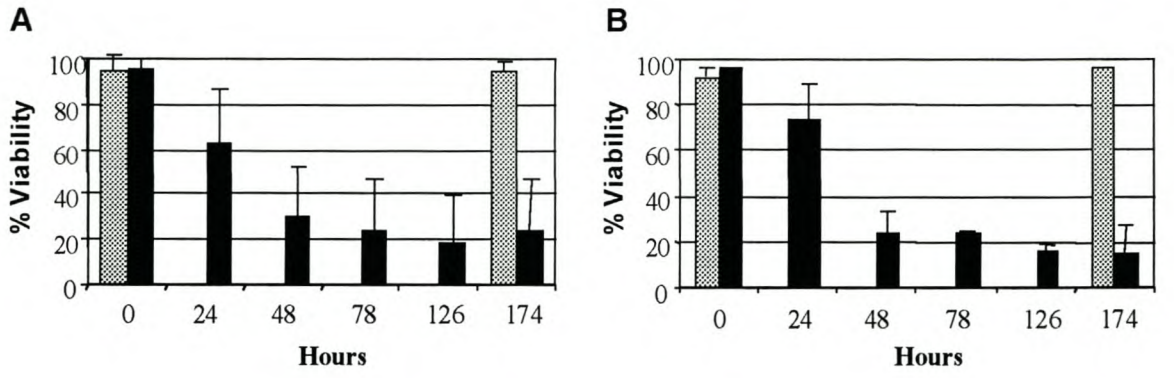


FIG 4.6 Heterogeneity (see sizes of standard deviation) in viability of biocide-treated biofilms (black) was greater within flow channels (A) than between duplicate experiments (B). In untreated biofilms [gray, sampled 1 h before (0 h) and 174 h after biocide treatment] viability values had low variability within and between duplicate flow channels.

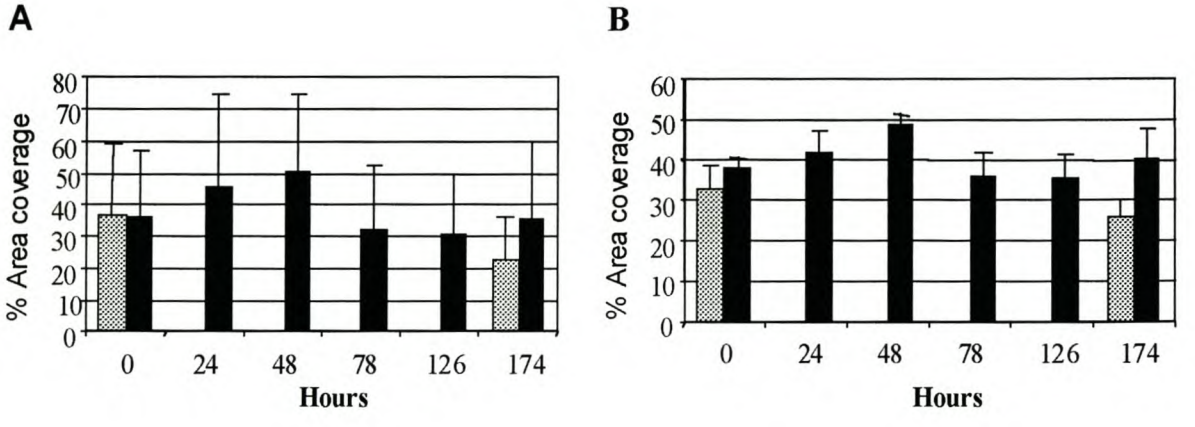


FIG 4.6.1 Heterogeneity in area coverage of biocide-treated biofilms (black) and untreated biofilms (gray), sampled 1 h before (0 h) and 174 h after biocide treatment was greater within flow channels (A) than between duplicate experiments (B).

4.6.1.1 Biofilm viability

As expected, the viability of biofilms cultivated with TSB (FIG 4.6.1.1.A) decreased sharply after a 5 h biocide treatment (0 h) when biocide treated biofilms (>10% viable) were compared to the untreated biofilms (99% viable). Biofilm viability started to recover within 24 - 78 h after biocide treatment. The high biocide susceptibility of TSB-grown biofilms correlated with previous results (Section 4.5). A full recovery, reaching original viability values (same as untreated biofilm at 174 h), was observed within the 7 day recovery period.

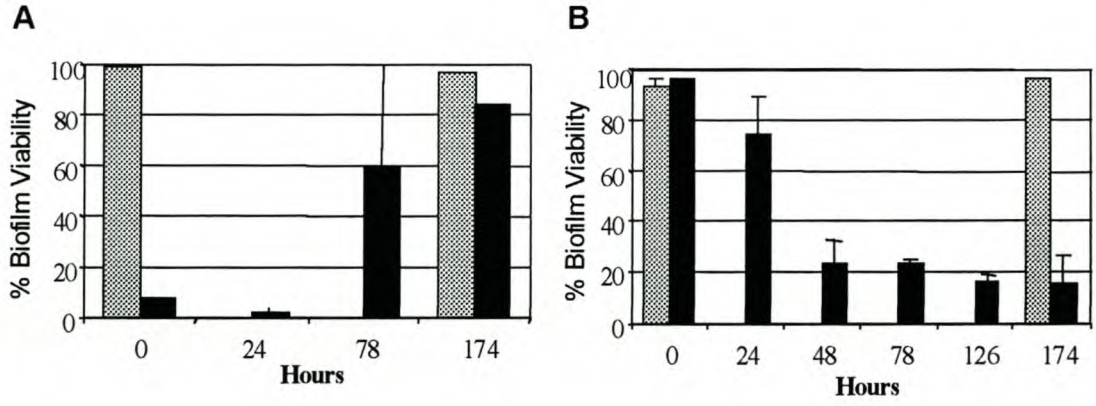


FIG 4.6.1.1 Biofilm susceptibility (in terms of viability) and recovery following biocide treatment (black bars) differed between the two nutrient conditions, 3.0 g/l TSB (A) and MSM + 0.1 g/l Glucose (B). Biofilms in untreated flow channels are shown in gray.

In contrast to the biofilms cultivated with TSB as nutrient, no decrease in biofilm viability was observed in biofilms grown on MSM + 0.1 g/l Glucose (FIG 4.6.1.1.B) within 1 h after biocide treatment, coinciding with the results obtained in previous experiments (Section 4.5). However, the marked decrease in biofilm viability over the subsequent 48 h showed that the perceived resistance observed in the previous experiments (Section 4.6) was short-lived and thus refuted the initial conclusion that the MSM + Glucose grown biofilm community had a high biocide resistance. The lack of biofilm recovery within seven days after biocide treatment suggests that the nutrient conditions were not favorable for cell repair and growth. The viability of the untreated biofilm remained stable over the recovery.

4.6.1.2 Biofilm area coverage

A decrease in area coverage from 40% to 25% was observed in biofilms cultivated on 3.0 g/l TSB, when treated with the biocide and observed over a 7-day recovery period (FIG 4.6.1.2.A). The same behavior was not observed in biofilms grown in MSM + Glucose (FIG 4.6.1.2.B). Nutrient conditions appear to impact biofilm area coverage, but not to such an extent as to biofilm viability.

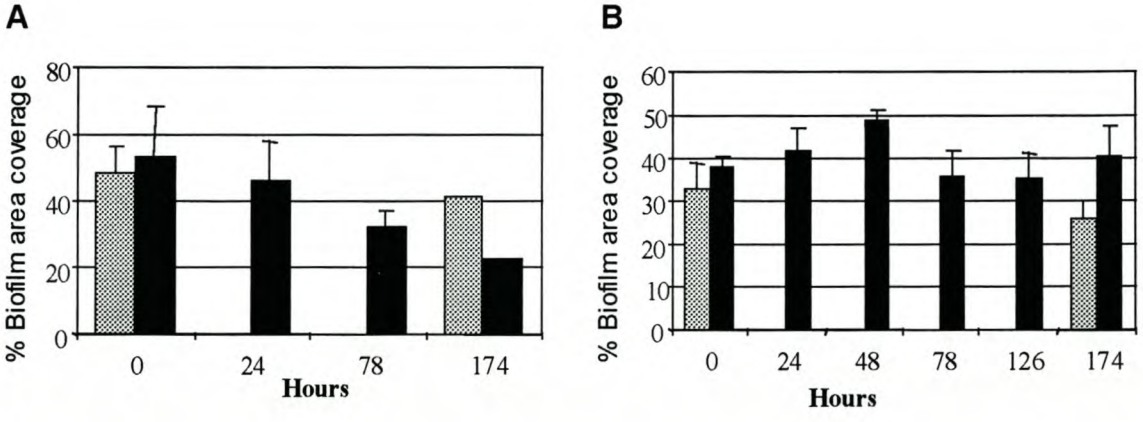


FIG 4.6.1.2 Area coverage of biofilms cultivated on 3.0 g/l TSB (A) and MSM + 0.1 g/l Glucose (B) following biocide treatment (black bars). Biofilms in untreated flow channels are shown in gray.

4.6.2 Analysis of the planktonic community

Although there was a notable variation in viability between flow channels at each sampling time (FIG 4.6.2.A), all replicate samples showed a marked decrease in viable cells after biocide treatment, followed by some degree of recovery. There was not an accompanying decrease in relative cell abundance, as shown in FIG 4.6.2.B.

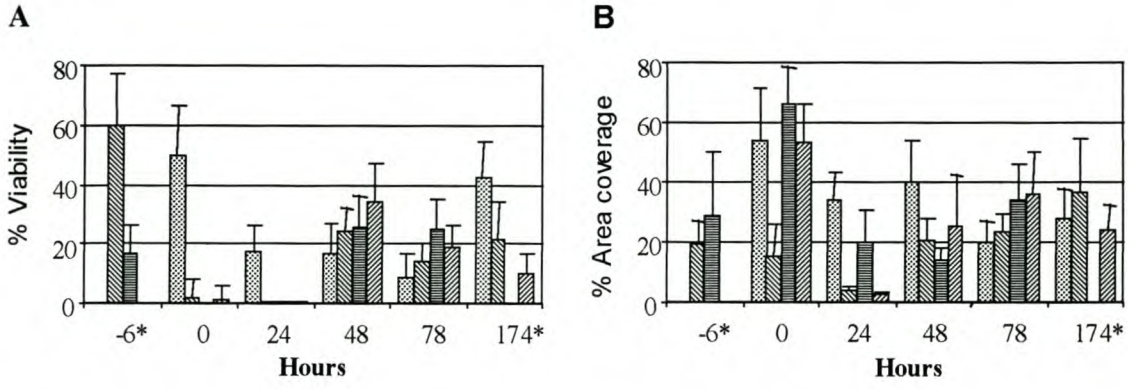


FIG 4.6.2 Recovery in planktonic cell viability (A) and relative abundance (B) (filter area coverage by total cell biomass) differed between samples collected from different channels at the same time. Untreated planktonic cells (gray) showed behavior similar to biocide treated planktonic cells (different stripe pattern for different flow channel samples) except for samples analyzed within 1 h after biocide treatment (0 h). Samples collected at –6 h represent planktonic communities from different flow channels 1 h before biocide treatment. These results show the variable nature of biofilm-derived planktonic cells (also see the similar behavior in FIG 4.6.2.1.

* Not all biocide-treated flow channels were sampled.

4.6.2.1 Planktonic cell viability

Viability of cells cultivated with TSB decreased from ~40% (untreated) to less than 10% within 1 h after biocide treatment (FIG 4.6.2.1.A). The decrease in cell viability continued for the next 24 h followed by a sudden increase in viability, reaching maximum values (~30%), within 48 h after biocide treatment. The planktonic cell viability under MSM + Glucose conditions (FIG 4.6.2.1.B) was close to 0% after biocide treatment (0 h). These results contradict previous observations made in Section 4.5.2.1, where effluent cells retained 50% viability. This may be due to possible changes in cell metabolic activity, growth rate, or nutrient conditions. Recovery in cell viability in these nutrient conditions occurred after 24 h reaching maximum values within 126 h.

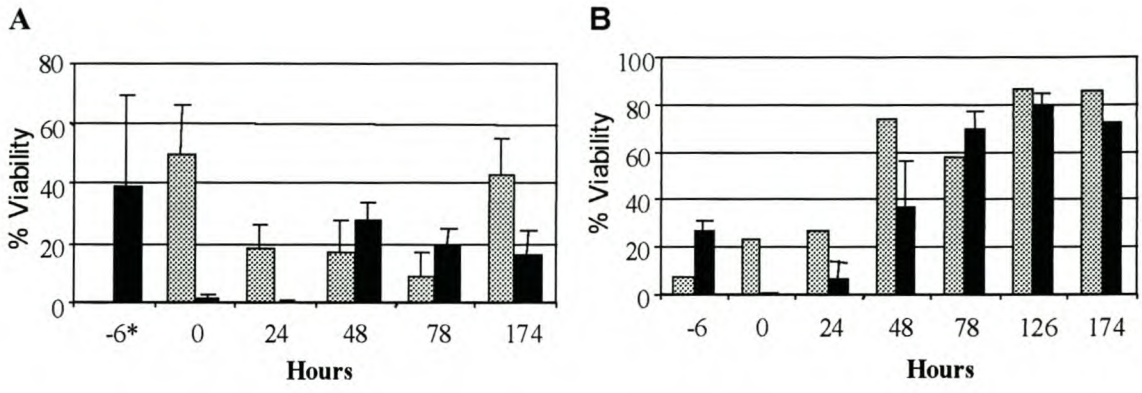


FIG 4.6.2.1 Average values for planktonic cell viability differed between the two nutrient conditions 3.0 g/l TSB (A) and MSM + 0.1 g/l Glucose (B). Untreated planktonic cells (gray) showed similar behavior as biocide treated effluent cells (black) except for samples analyzed within 1 h after biocide treatment (0 h). Samples collected at –6 h represent the planktonic cell viability 1 h before biocide treatment.

*Control flow channel was not sampled.

4.6.2.2 Planktonic cell abundance

There was no difference in cell abundance in the effluents of treated and untreated TSB-grown biofilms (FIG 4.6.2.2.A) within 1 h after biocide treatment, while a decrease in cell abundance was observed in the treated channels within the following 24 h. After 30 h, cell abundance started to recover, reaching maximum values (30%) within 78 h after biocide treatment. The recovery rate of associated culturable cell numbers appeared to be slightly faster (24 h) than the relative cell abundance.

In contrast to TSB, there was a notable decrease in cell abundance in the MSM + Glucose-fed flow channels within 1 h after biocide treatment (FIG 4.6.2.2.B). It appeared that the biocide inhibited the release of biofilm cells directly after biocide treatment. Relative cell abundance in the effluent only started to increase within 78 h of the recovery period. When comparing the recovery pattern of culturable cells number with the relative cell abundance, it was found that the recovery rate of culturable cell numbers was faster than that of relative cell abundance.

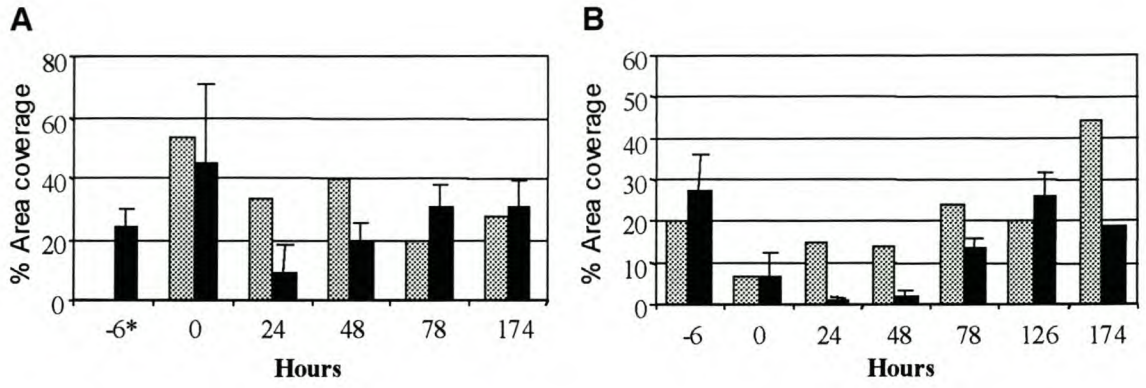


FIG 4.6.2.2 Relative planktonic cell abundance (filter area coverage by total cell effluent biomass) recovery differed between the two nutrient conditions, 3.0 g/l TSB (A) and MSM + 0.1 g/l Glucose (B). Untreated planktonic cells (gray) showed similar behavior as biocide treated cells (black) except for samples analyzed within 1 h after biocide treatment (0 h). Samples collected at –6 h represent the planktonic cells 1 h before biocide treatment.
*Control channel was not sampled.

Since it is assumed that most effluent cells were derived from the biofilm community, it was proposed that cells at the outer regions of biofilms react differently towards biocide treatment under different nutrient conditions.

4.6.2.3 Culturable cell counts

The cell viability recovery pattern (FIG 4.6.2.1A) under 3.0 g/l TSB conditions showed a similar trend than the recovery in culturable cell numbers (FIG 4.6.2.3.A) from the same sample. One hour after biocide treatment, no culturable cells were detected in the effluent (100% killing). Cell numbers recovered within the following 48 h to original values (10^8 CFU/ml) and remained stable for the remaining recovery period. The number of culturable planktonic cells under MSM + Glucose conditions (FIG 4.6.2.3.B) decreased to about a 1000 cells/ ml just after biocide treatment (99.99% killing). Full recovery to original cell numbers was observed within three days after biocide treatment. Planktonic populations that originated from TSB-grown biofilms were more susceptible than cells originating from biofilms grown on MSM + 0.1 g/l Glucose. Untreated planktonic cell numbers in both nutrient conditions remained reasonably stable over the seven-day observation period (FIG 4.6.2.3).

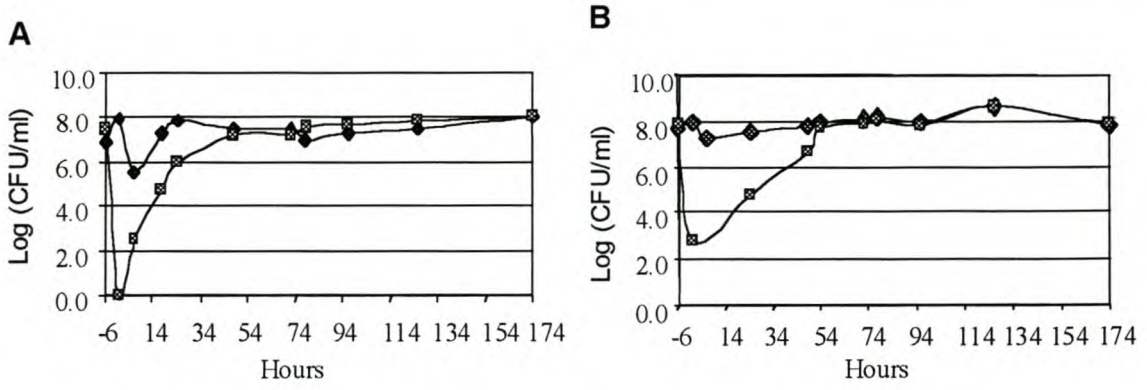


FIG 4.6.2.3 Susceptibility and recovery of planktonic cells differed between the two nutrient conditions, 3.0 g/l TSB (A) and MSM + 0.1 g/l Glucose (B). Compared to biocide treated planktonic cells (square), untreated planktonic cell (diamond) numbers remained stable over time.

4.6.3 Proposed model of biofilm-planktonic interactions

A stable biofilm community is often characterized by stable effluent cell numbers over time (Sauer et al 2002). Since stable and high cell numbers in the untreated effluent (FIG 4.6.2.3) of both nutrient conditions were observed over the recovery period, it was assumed that the 3-day-old mixed-species biofilm community had stabilized before biocide treatment was applied. It was also assumed that the majority of planktonic cells were derived from the biofilms and not from planktonic growth. The latter assumption was based on the fact that the dilution rates applied in the flow channels were up to 34 times higher than the highest specific growth rates of the bacterial isolates (Section 4.3.3 and 4.3.4). Stable culturable cell numbers and biofilm viability in both nutrient conditions suggested that the biofilm communities were metabolically active and grew with a rapid cell turnover. From this it can be suggested that biofilm growth was not limited by the low nutrient condition of MSM + 0.1 g/l Glucose (FIG 4.6.2.3.B) prior to biocide treatment.

Microbial communities (biofilm and planktonic growth form) that were grown in the complex TSB were highly susceptible towards the biocide, with biofilm communities (FIG 4.6.1.1.A) being only slightly more resistant than their associated planktonic communities (FIG

4.6.2.1.A). These observations are in contradiction to what is often claimed in the literature (e.g. Brown et al 1988; Nichols 1989). Biofilm and planktonic communities recovered fast in the TSB medium, with the recovery rate of the planktonic community being faster than that of the biofilm community. One hour after biocide treatment, the number of cells (area covered on filter) did not differ from the untreated controls (FIG 4.6.2.2.A). However, less than 10% of these treated planktonic cells were viable (FIG 4.6.2.1.A) and none were culturable (FIG 4.6.2.3.A). This phenomenon of microbial cells being viable, but non-culturable has attracted an increased interest, since molecular techniques proved the presence of microbes in environmental samples that could not be cultured (Amann et al 1995). Interestingly, as the abundance of treated planktonic cells (of which < 5% were viable) deceased over the following 24 h, culturable cell numbers increased to 10^6 CFU/ml. A plausible explanation may be that the majority of planktonic cells were biofilm cells damaged by the biocide, which had been able to recover sufficiently to regain their culturability, yet appeared non-viable. It is known that the one biocide component, glutaraldehyde, interacts with the microbial cell membrane (McDonnell and Russell 1999). Therefore, cell membranes could have been damaged in such a way that the fluorescent probe was able to penetrate the cell, while cell repair mechanisms were able to restore cell culturability and prevent cell death.

Microbial communities supplied with MSM + Glucose showed a different antimicrobial susceptibility and regrowth behavior. Initially, (0 h after biocide treatment) the biocide appeared to have no impact on the biofilm viability (FIG 4.6.1.1.B), yet the planktonic relative cell abundance (FIG 4.6.2.2.B) and viability (FIG 4.6.2.1.B) had decreased drastically at that time. This was then followed by a decrease in biofilm viability, which did not recover within the next seven days, while within that period planktonic cell numbers fully recovered. The lack of recovery in the biofilms suggests that the nutrient conditions were not favorable (incomplete) for cell repair and growth. While biofilm viability did not recover, planktonic cell viability (FIG 4.6.2.1.B), relative abundance (FIG 4.6.2.2.B) and culturable cell numbers (FIG 4.6.2.3.B) increased after three days (78 h).

Culturable cell numbers in the TSB medium recovered to original values within 48 h, and in the MSM + Glucose medium within 78 h after biocide treatment. In each instance, this was followed by an increase in planktonic cell viability and relative abundance. From the

planktonic cell behavior (cell culturability, cell abundance and viability) it can be expected that the biofilm community also recovered. Surprisingly, this was not the case, as recovery in biofilm viability was observed much later. This raises the question as to the source of the high numbers of planktonic cells, especially when biofilm viability had not yet recovered and biofilm area coverage had not changed notably. To provide possible answers to this question, a model based on the experimental observations was proposed.

In the model (FIG 4.6.3), it is hypothesized that cell recovery occurs primarily at the biofilm – bulk-fluid interface. Initially, dead and damaged (non-culturable) cells are released, which is then followed by a period of biofilm cell repair and regrowth in this zone (low relative cell abundance in effluent). During this latter period, recovering cells, as well as newly formed daughter cells detach from the biofilm surface at high rates, while the rate of recovery among the permanently attached cells in the deeper zones of the biofilm is much slower, or even non-existent. Recovery in the deeper zones is thus to a large degree dependent on the rate at which the dead cells trapped in the biofilm matrix is lysed and degraded, or may be hindered by the presence of biocide residues immobilized in the matrix. It is possible that detachment of viable cells at the outer region is stimulated by a lack of essential nutrients needed for biofilm maintenance, or more likely, detachment provides a mechanism to minimize competition for nutrients. Thus, it is possible that recovery and active growth at the biofilm surface is responsible for the rapid recovery in planktonic cell numbers. Nutrient gradients within the biofilm may also contribute to slower biofilm recovery.

Based on the observation that recovery of planktonic cell numbers occurred faster than the recovery of biofilm viability, it is hypothesized that the outer surface of a biofilm is primarily responsible for planktonic cell abundance, irrespective of the overall biofilm viability or activity. This challenges the hypothesis that a stable planktonic population represents a stable biofilm community (Sauer et al 2002). Stable planktonic cell abundance could therefore only give an indication of active biofilm surface growth.

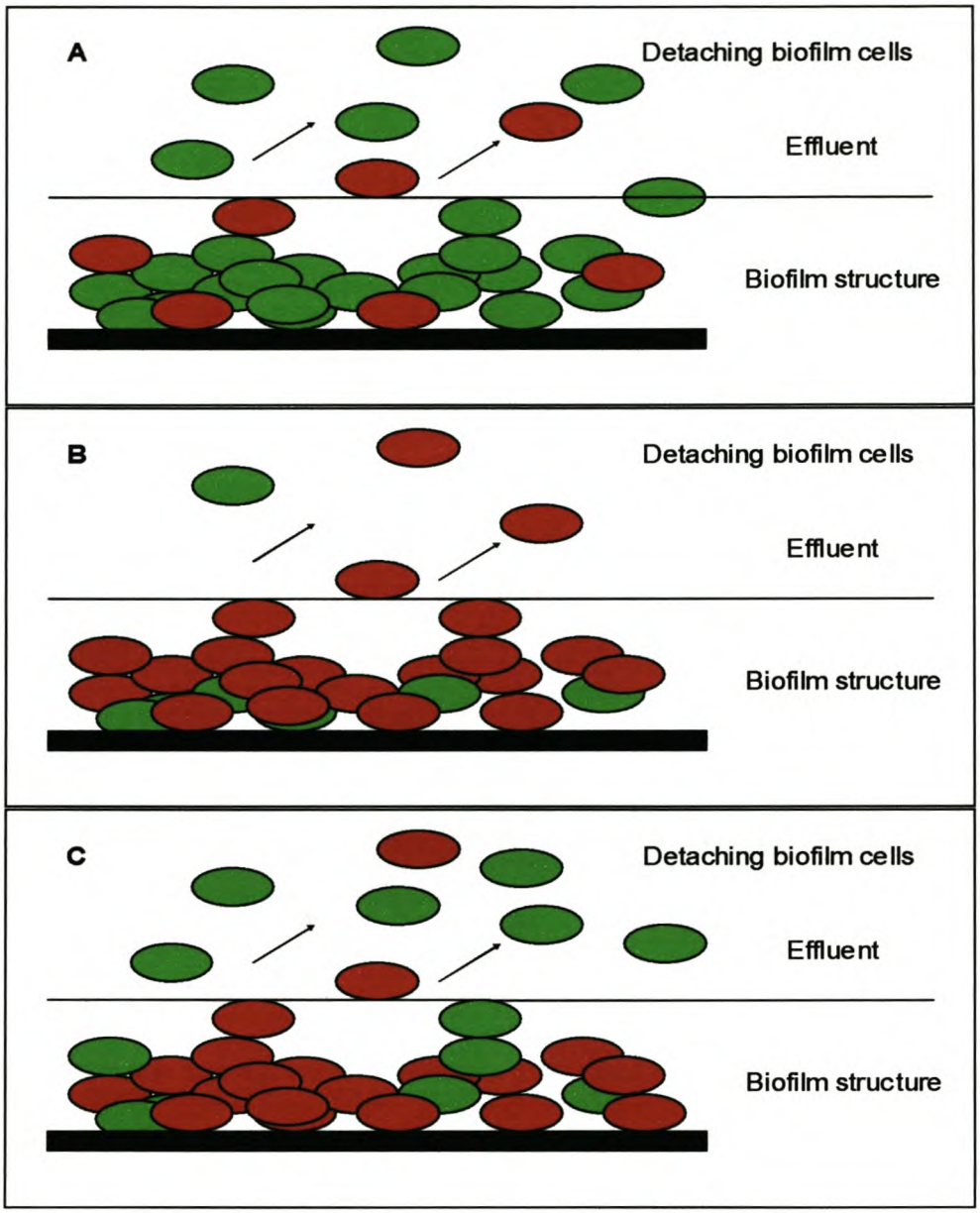


FIG 4.6.3 A model proposed to explain the faster recovery of the planktonic community compared to the biofilm community. This model proposes that most effluent cells are biofilm-derived under conditions of high flow (dilution rate). Dark cell represent the non-viable or biocide damaged cells, while the light cell are viable cells. When a stable biofilm community (A) is treated with a biocide (B), planktonic cell abundance decreases and the majority of the biofilm cells die, which is then followed by the recovery of effluent cell numbers first (C) and then followed by biofilm recovery (A).

4.7 POPULATION DYNAMICS

Population dynamics, in terms of variation in the presence and relative ratios of 4 bacterial isolates (CT01, CT03, CT04 and CT07) in the flow channel effluent were determined. Community responses were studied under two different experimental conditions. Firstly, the influence of six different nutrient conditions on the presence and relative abundance of the bacterial isolates was investigated after three days growth in a flow cell microcosm. Secondly, the influence of biocide treatment on biofilm-associated planktonic populations, grown under two different nutrient conditions, was observed over a seven-day recovery period. Three different methods were applied, conventional spread plating, terminal restriction fragment length polymorphism (T-RFLP) and fluorescent *in situ* hybridization (FISH). The two culture-independent techniques, T-RFLP and FISH, were applied to the second experimental condition for comparison of the results obtained from the conventional culture-dependent technique. This work was performed with the assumption that, although the planktonic populations in flow cell effluent were derived from the respective biofilm communities they might not necessarily represent the community structure of biofilms.

4.7.1 Conventional plate count technique

4.7.1.1 Influence of nutrient conditions

The culturable cell counts differed between the different nutrient sources (FIG 4.7.1.1.1). Under both nutrient conditions (TSB and MSM + Glucose), planktonic cell numbers increased with an increase in nutrient concentration. Numbers in the effluent exceeded those in the original inoculum only when grown in 3.0 g/l TSB. The behavior of the different isolates did not always corresponded with the general trend of the total cell count. Isolates CT03 and CT07 increased in cell numbers as nutrient concentrations increased. Being the more dominant isolates they influenced the trend, whereas isolates CT01 and CT04 were present in lower numbers and had no influence on the general trend. When TSB was provided as nutrient, the culturable cell count of isolate CT01 was the highest in 0.3 g/l TSB and lowest at 3.0 g/l TSB. Isolate CT04 preferred 0.3 g/l TSB, and for all isolates no difference in cell numbers between MSM + 1.0 g/l and 0.1 g/l Glucose was observed. Isolate CT03 was the dominant population in the MSM + 0.0 g/l Glucose flow channels, while

isolates CT04 and CT07 were present in the lowest numbers. The optimal nutrient condition for all four isolates were 0.3 g/l TSB and 3.0 g/l TSB, while the weakest growth was observed in MSM without glucose, as expected. In the latter nutrient condition, the overall cell numbers were surprisingly high (10^6 CFU/ml). The bacterial isolate with the widest nutrient range was isolate CT03 followed by CT01 and CT07 and then finally isolate CT04 having the most specified range.

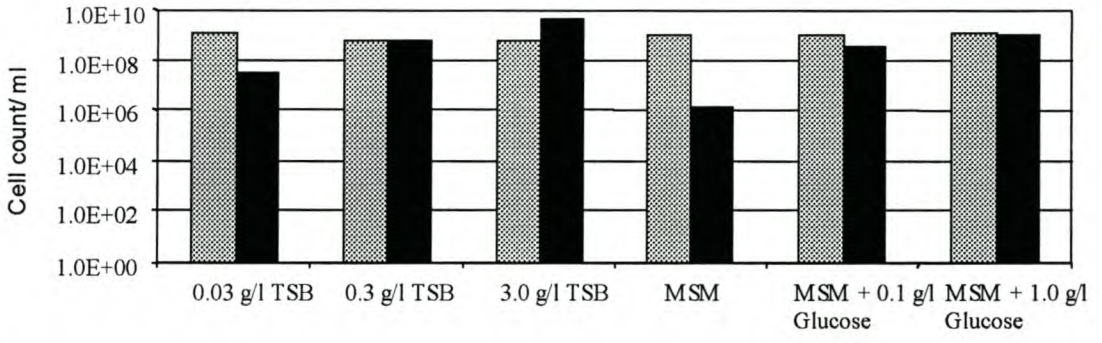


FIG 4.7.1.1.1 Planktonic culturable cell numbers (black) changed between the different nutrient conditions (TSB and MSM + Glucose) when compared to the inoculation cell numbers (gray) after three day.

The ratio of the inoculation mix varied between samples (FIG 7.1.1.1.2.A). Two days growth under different nutrient conditions caused a change in isolate ratio (FIG 7.1.1.1.2.B). Under TSB, the relative abundance of isolate CT03 increased as nutrient concentrations increased, while CT01 decreased. Isolate CT07 was present at lower numbers than CT03 at all three TSB concentrations. Under the different glucose concentrations, the relative abundance of isolate CT03 increased as glucose concentrations decreased, while the abundance of CT07 increased with an increase in glucose concentrations. The number of isolate CT01 relative to the other isolates slightly increased as glucose concentrations decreased. The numbers of CT04 were too small for detection.

Even though the ratio of isolate numbers in the inoculum differed, a distinct population ratio developed for each of the different nutrient conditions, which may have been influenced by the ability of each population to respond and utilize the various nutrient sources. A decrease or increase in the relative abundance of an isolate when compared to the other isolates, did not

always positively correspond with the changes observed in the respective culturable cell counts. The presence and ratios of the four isolates within the effluent sample three days after inoculation were clearly influenced by not only the difference in nutrient type, but also by the nutrient concentrations.

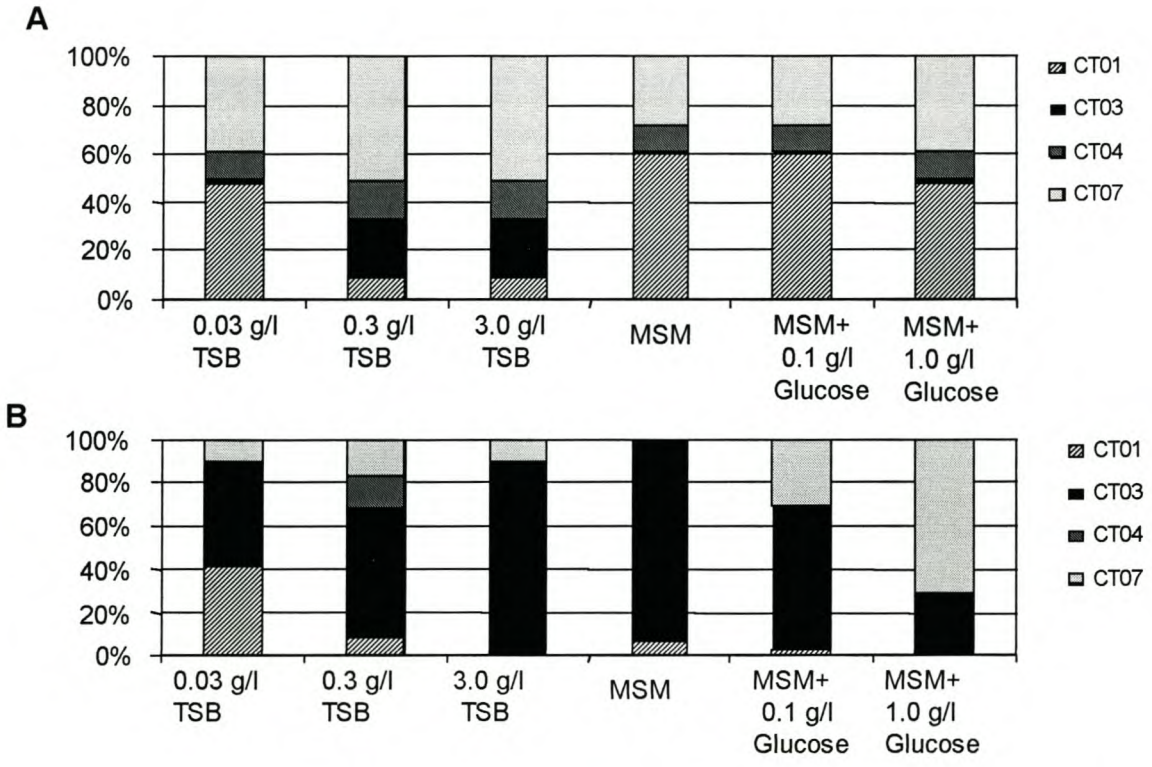


FIG 4.7.1.1.2 Influence of nutrient sources on the species composition of the planktonic community (B) after being exposed to different nutrient conditions. The initial composition of the inoculum (A) changed depending on the nutrient source and concentrations. Under most nutrient conditions, isolate CT03 increased in relative abundance, whereas the numbers of isolates CT01, CT04 and CT07 decreased when compared with their respective inoculation ratios.

4.7.1.2 Influence of biocide treatment

Culturable cell numbers of the four different isolates in the untreated effluent of both nutrient conditions stayed relatively stable (FIG 4.7.1.2.1. B and D). Biocide treatment had a marked impact on the rate of recovery by the bacterial populations. This could clearly be seen with population CT04, which did not recover when grown in 3.0 g/l TSB, while growing in MSM + Glucose (FIG 4.7.1.2.1 A and C), recovery in numbers was observed after some time. Both isolates CT01 and CT04 were not detected 1 h after biocide treatment (0 h). Isolate CT01 started to recover within 6 - 48 h after biocide treatment under both nutrient conditions, while isolate CT04 only recovered after 4 days after biocide treatment when grown within 0.1 g/l MSM + Glucose. Under both nutrient conditions, isolate CT01 recovered slower than CT03 and CT07, without reaching original numbers within the recovery period.

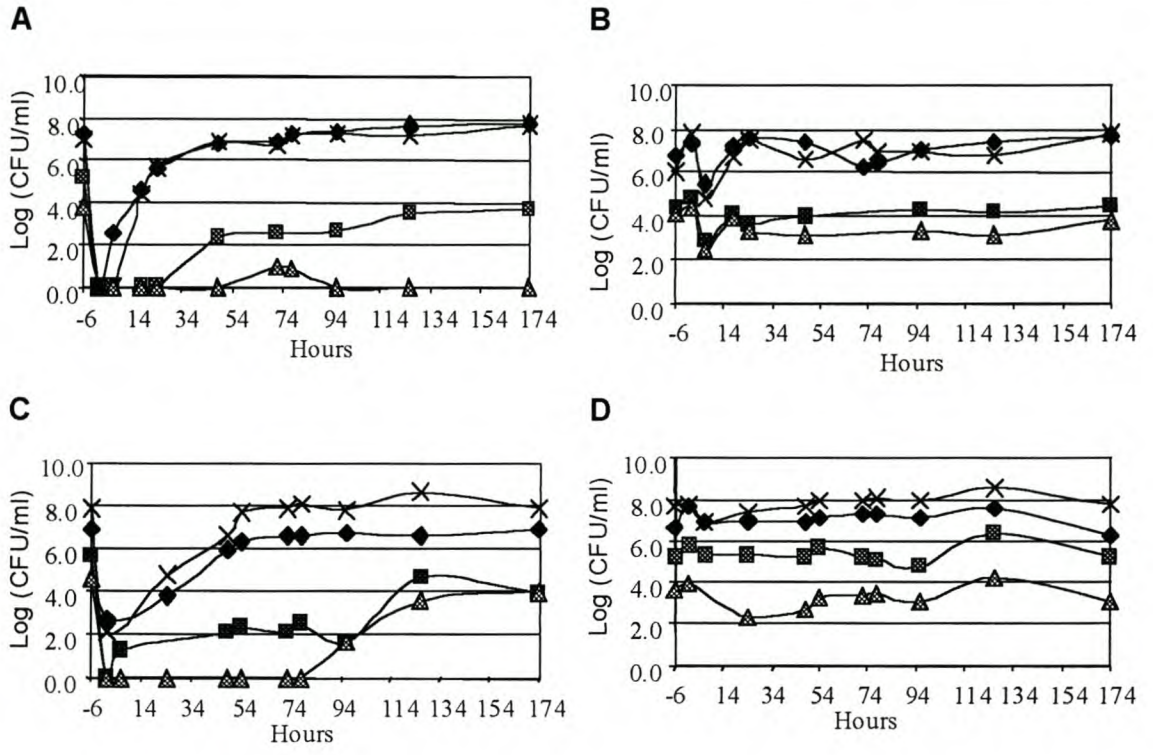


FIG 4.7.1.2.1 Planktonic population dynamics of biocide treated (A and C) and untreated (B and D) flow channel effluent. Nutrient conditions, 3.0 g/l TSB (A and B) and MSM + 0.1 g/l Glucose (C and D), influenced the recovery of bacterial isolates, CT01 (square), CT03 (cross), CT04 (triangle) and CT07 (diamond) after biocide treatment, while isolate cell numbers in the untreated effluent remained stable. Nutrient condition and biocide treatment had the greatest impact on isolate CT04.

It needs to be confirmed whether the disappearance of isolate CT04 from the planktonic community reflected a loss of that population from the respective biofilm community. It would probably not be the case, since the phenomenon of viable but non-culturable cells has been observed previously, and also the recovery in cell numbers might have required a longer period than allowed in this study. Isolate CT04 might have survived within deeper parts of the biofilm, but was killed closed to the bulk-fluid interface, thus explaining its absence from the planktonic community. Similar cell behavior was observed previously by Banks and Bryers (1991) and Garland et al (2001).

The untreated planktonic community under TSB conditions showed greater variation in the relative abundance of the individual isolates compared to the treated planktonic community (FIG 4.7.1.2.2.A). It appeared that biocide treatment had a stabilizing effect on the two dominant isolates CT03 and CT07, which were present in equal cell ratios (FIG 4.7.1.2.2.B). Isolate CT07 was the dominant population within the untreated planktonic community. In untreated planktonic communities grown in MSM + 0.1 g/l Glucose, the relative abundance (FIG 4.7.1.2.2.D) of isolate CT03 regained it's dominance during the 7-day sampling period after an unexplained initial decrease in its dominance at 0 h (end of biocide treatment). Likewise, isolate CT03 was also found to be the dominant bacterial population in the treated planktonic community (FIG 4.7.1.2.2.C), except directly after biocide treatment (0 h). Isolate CT07 was less susceptible than CT03, but it was not able to use this advantage to gain dominance over CT03, for within 24 h CT03 had regained its dominance. Changes in cell numbers of isolates CT01 and CT04 were only detected with conventional spread plating.

Biocide treatment and nutrient condition had an impact on the dominance, survival and recovery of the different bacterial populations within the planktonic community. A shift in the community structure, when grown in TSB, was observed when isolate CT07 lost its dominance. Even though bacterial populations were more susceptible to biocide their recovery rate was faster, than population grown in MSM + Glucose, with the exception of isolate CT04. Isolate CT04 did not recover under TSB conditions, while a delayed recovery to original values was observed within 7 days after biocide treatment under MSM + Glucose conditions.

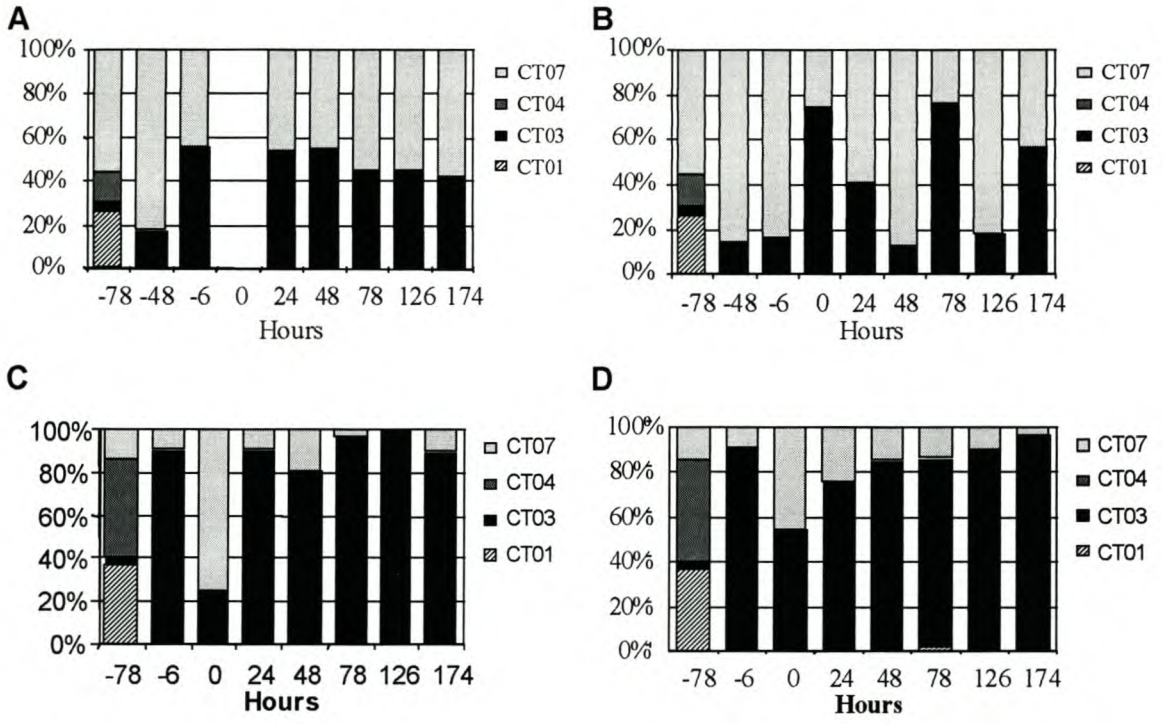


FIG 4.7.1.2.2 Relative abundance of planktonic populations differed between nutrient conditions, 3.0 g/l TSB (A and B) and MSM + 0.1 g/l Glucose (B and D), over time. Difference in bacterial isolate relative abundance was also observed between biocide-treated (A and C) and untreated (B and D) planktonic communities. Isolates ratios of CT01and CT04 were not detected due to their low cell numbers or due their absence from the planktonic community.

4.7.2 Terminal restriction fragment length polymorphism (T-RFLP)

T-RPLP was used to confirm the plate count results on planktonic community dynamics (Section 4.7.1) after biocide treatment. Application of quantitative analysis was not attempted with this technique, as there are still limitations in that regard (Liu et al 1997; von Wintzingerode et al 1997; Dunbar et al 2001). However, qualitative analyses of T-RFLP have become a useful tool to study diversity, structure and dynamics of microbial communities (Liu et al 1997; Blackwood et al 2003; Lueders and Friedrich 2003).

A terminal restriction fragment (T-RF) of each bacterial isolate was determined by analyzing 16S rDNA from each isolate. As two restriction enzymes, *Alu*I and *Rsa*I, were used

separately, two T-RF's for each labeled primer were obtained per isolate. A *Rsa*I restriction site was found within the sequence of the reverse primer (Hex-labeled) and therefore, theoretically no T-RF's could be obtained. T-RF's of each isolate are presented in Table 4.7.2. No marked difference between predicted and determined T-RF's was found. In pure culture, one dominant peak with several smaller peaks was obtained representing the T-RF of each bacterial isolate (Appendix: 4.7.1). In contrast, when the four test isolates were mixed in equal numbers before DNA isolation, peaks of CT03 and CT07 dominated when compared with those of CT01 and CT04 (FIG 4.7.2.1, Appendix: FIG 4.7.2).

Table 4.7.2. Predicted and analyzed terminal restriction fragment lengths of the four selected bacterial isolates, determined by T-RFLP analysis of planktonic communities.

Primer	Forward primer F341-FAM		Reverse primer R1389-HEX	
Restriction enzyme	*Alu*I	*Rsa*I	*Alu*I	*Rsa*I
CT01	214 bp	123 bp	340 bp	None
CT03	~505 bp *505 bp	~550 bp *550 bp	128 bp *129 bp	None
CT04	304 to305 bp *304 bp	139 bp *140 bp	69 bp *44 bp	None
CT07	308 bp	Larger than 500 bp	128 bp *131 bp	None

*Predicted TRF's with application of the software-based program DNAMAN and partially known 16S rDNA sequences. Reverse primer R1389-HEX contained an *Rsa*I restriction enzyme cutting site. Values with ~ (>500) were estimated because a 500 bp size standard was used.

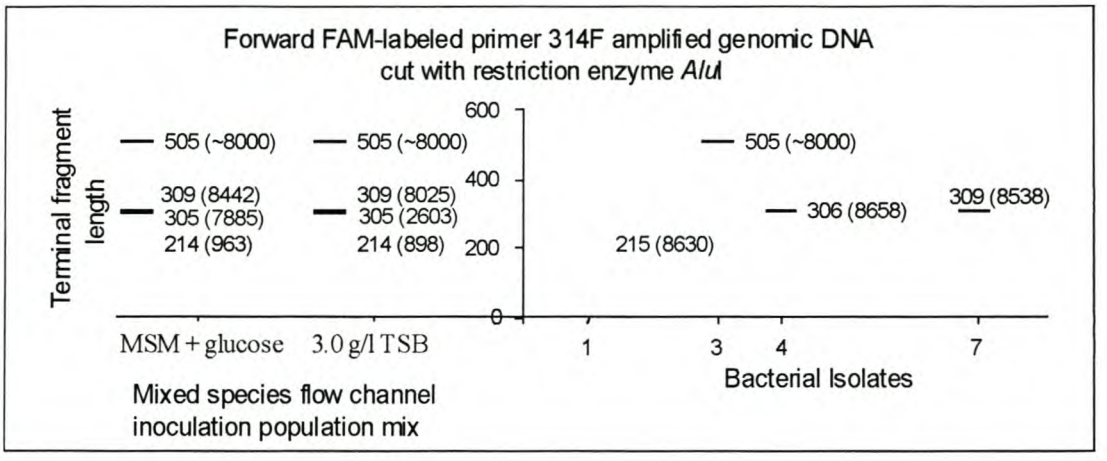


FIG 4.7.2.1 Terminal restriction fragment lengths of the four selected bacterial isolates (CT01 (1), CT03 (3), CT04 (4) and CT07 (7)) with their respective peak heights indicated in brackets. Peak heights of pure isolates were all detected in high intensities (peak height), while mixed-species cultures (MSM and TSB) with equal bacterial numbers showed clear selection of isolates CT03 and CT07. Values with ~ (>500) were estimated because a 500 bp size standard was used.

Even after a number of optimization steps, the peak heights of CT01 and CT04 did not increase, indicating that either DNA isolation or PCR bias (e.g. primer specificity, annealing temperatures) had occurred. More likely, it was the case that DNA isolation bias may have contributed to the preferential PCR selection of CT03 and CT07. T-RF's of isolates CT01 and CT04 were clearly detectable in the inoculum, while no detection in the planktonic community collected three days later (-6 h in FIG 4.7.2.2), could be made (FIG 4.7.2.2). Within all planktonic communities analyzed, biocide-treated (FIG 4.7.2.2 B and D, FIG 4.7.2.4) or untreated (FIG 4.7.2.2 A and C, FIG 4.7.2.3), only CT03 and CT07 were detected over the experimental period. The community profiles obtained from the two nutrient conditions did not differ significantly. This contradicts the results obtained by the conventional culture technique (FIG 4.7.1.2.1. B and D) where isolates CT01 and CT04 were present in low number within the untreated community. The lack of detection of CT01 and CT04 could be explained by PCR bias, which might have been enhanced further by the low cells number within the respective planktonic communities.

Despite the presence of extra peaks observed in the electropherograms for pure cultures and planktonic communities, which complicated accurate analyses, it was possible to obtain repeatable community profiles. It is likely that extra T-RF's formed as a result of incomplete restriction enzyme digestion, or the formation of partially single-stranded fragments (pseudo-T-RF's), which are typically not properly digested by restriction enzymes. The latter reason was only recently discovered by Egert and Friedrich (2003). The authors proposed that pseudo-TRF's could be eliminated by an extra purification step, using a mung-bean enzyme after the restriction enzyme digestion. In this study, T-RFLP was less sensitive in revealing changes in community composition than conventional spread plate technique.

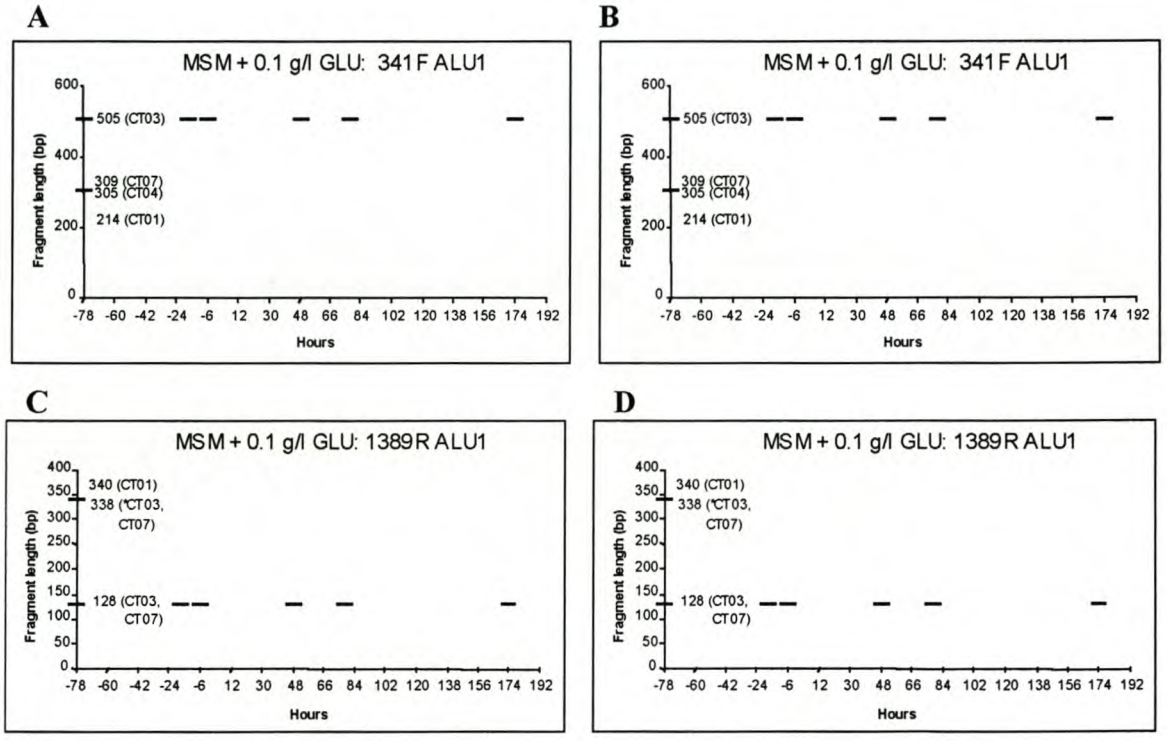


FIG 4.7.2.2 Terminal restriction fragment lengths, which identified bacterial isolates within the planktonic communities collected at different times after biocide treatment, did not change. The results obtained from the two primers, forward primer 341F (A and B) and reverse primer 1389R (C and D) cut with the restriction enzyme *Alu*I, complemented each other. Untreated (A and C) and treated (B and D) planktonic communities were analyzed. All isolates were detected in the inoculum at –78 h, while only isolates CT03 and CT07 were detected at later stages of the experiment. Even after optimization of the technique, bacterial isolates CT01 and CT04 were not detected.

* Secondary T-RF of isolates CT03 and CT07.

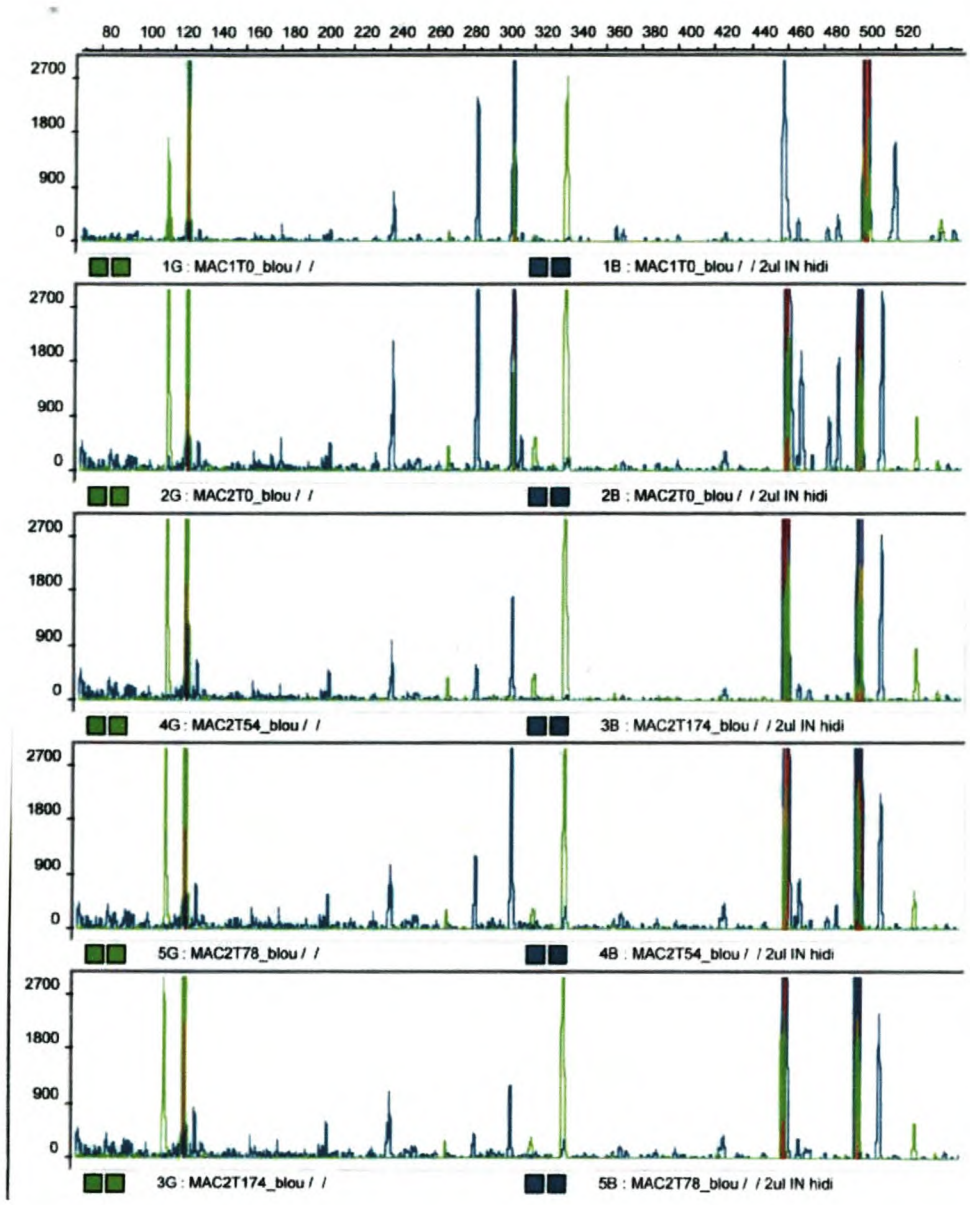


FIG 4.7.2.3 The T-RFLP community profile of the untreated planktonic community (C2) grown under MSM + 0.1 g/l Glucose (M) did not change over time (T= hours after biocide treatment). The blue peaks represent the terminal restriction fragments obtained when using the FAM-labeled forward primer (341F), while the green peaks represent the terminal restriction fragments obtained when using the HEX-labeled reverse primer (1389R). Both were cut with the restriction enzyme *Alu* I (A).

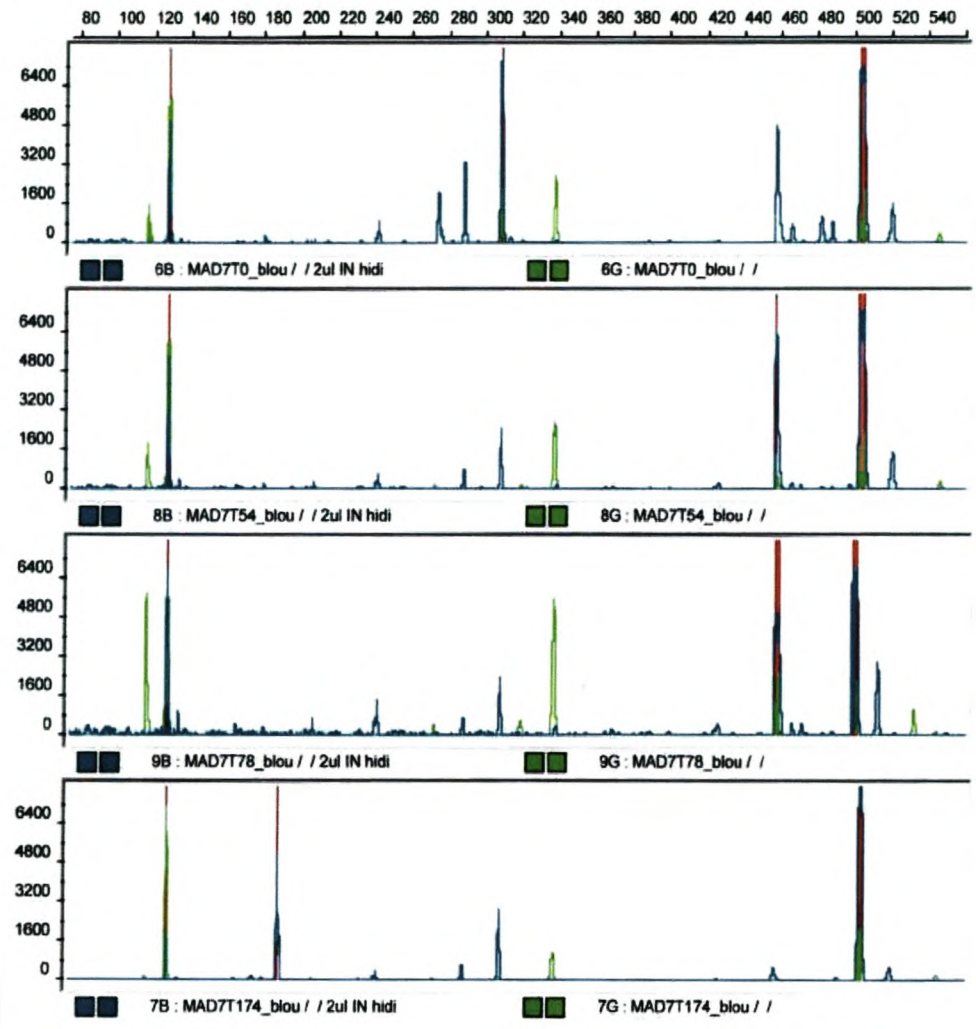


FIG 4.7.2.4 The T-RFLP community profile of the biocide treated planktonic community grown in MSM + 0.1 g/l Glucose (M) showed little change over time (T= hours after biocide treatment). The blue peak represents the terminal restriction fragments obtained when using the FAM-labeled forward primer (341F), while the green peaks represent the terminal restriction fragments obtained when using the HEX-labeled reverse primer (1389R). Both were cut with the restriction enzyme *Alu* I (A).

4.7.3 Fluorescent *in situ* hybridization (FISH)

While cultivation techniques showed the presence of CT04 in the planktonic communities grown on MSM + 0.1g/l Glucose, this isolate was not detected by T-RFLP. Therefore, FISH was applied to confirm the presence of isolate CT04. A species-specific primer was designed for the *in situ* identification of CT04. Optimizations and evaluation steps confirmed probe specificity (FIG 4.7.3).

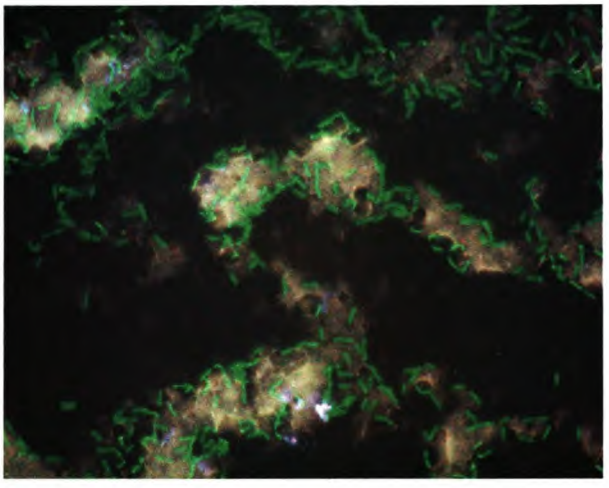


FIG 4.7.3 The fluorescein-labeled oligonucleotide probe was specific for isolate CT04 (green cells). In a mixture with CT01, CT03 and CT07, isolate CT04 was clearly visible by green fluorescence, while the other cells were recognized by their slight reddish-blue autofluorescence, or blue when counterstained with DAPI (not shown).

Applying FISH to the planktonic communities (biocide treated and untreated) of both nutrient conditions did not result into the detection of CT04. Detection of the fluorescent-labeled CT04 cells was hampered especially in the biocide treated samples by autofluorescent cells, which probably were caused by the biocide component glutaraldehyde. Previous fixation experiments showed that when using glutaraldehyde as fixative agent, fixed cells showed strong autofluorescence. The weak and fast fading fluorescence of the probe might also have contributed to the lack of detection of isolate CT04. Another reason for the lack of conclusive results could have been the ethanol fixation method chosen to preserve effluent samples for later analysis. Ethanol fixation was initially chosen for the similar results obtained when

compared with the paraformaldehyde fixation method, but at that stage, the isolate-specific probe had not been designed and the probe used was labeled with a different fluorescent molecule.

It is recommended that for future work, the probe be redesigned with a small shift in the 16S rRNA gene sequence position (Behrens et al 2003) and labeled with a stronger and more stable fluorescent marker, e.g. CY3.

4.7.4 Conclusion

Nutrient conditions and biocide treatment had an influence on the composition of the defined mixed-species planktonic community that was present in flow channel effluents. However, changes observed in the planktonic community do not necessarily imply identical changes in the associated biofilm community, and therefore can only indicate that some form of alteration in the biofilm composition may have taken place.

Changes in species richness (number of species within the community) and evenness (size of species population within the community) were observed, when applying a conventional culture technique to monitor population dynamics. In contrast, results obtained by T-RFLP analyses, proposed that an initial loss of isolates CT01 and CT04 occurred before biocide treatment, which was then followed by no further changes in community composition after biocide treatment for the remaining experimental period. In addition, the presences of isolate CT04 in the effluent collected three days after inoculation could not be confirmed with FISH. Even though literature proposes that culture-independent techniques are superior to cultivation techniques (Amann et al 1995), these results suggest that cultivation techniques remain a valuable tool for studying microbial diversity and structure in environmental samples.

CHAPTER 5:
GENERAL CONCLUSION

The influence of biocide treatment on biofilm and planktonic communities, initially observed within an operational cooling tower system, was confirmed with subsequent laboratory experiments. Heterogeneity in biofilm and planktonic viability and abundance was observed, which emphasized the importance of statistically representative sample size. Using a statistical formula, it was determined that average sample sizes of 40 - 60 images for glass slides, 30 – 50 images for flow cells and 20 – 30 images for filters, were required. Furthermore, after considering the variety of factors that might have influenced the results obtained from the cooling system, it was decided to perform subsequent experiments in a small-scale flow system. This allowed greater control over environmental factors such as community complexity, nutrient conditions, temperature, and biocide application. Seven bacterial isolates from cooling tower water were isolated and characterized based on their phenotypic differences. Four of the seven isolates were identified based on their partially sequenced 16S rDNA sequences. These four isolates, which included two *Pseudomonas* species (CT03 and CT07), a *Dyadobacter* species (CT04) and an unidentified strain (CT01), were used for studying antimicrobial susceptibility and population dynamics in a defined biofilm community under different nutrient conditions.

Planktonic numbers in replicate flow channel effluents reached steady state within 3 days after inoculation, suggesting that the biofilm community stabilized during this period. It was proposed that microbial cells from the effluent were mostly biofilm derived, and not due to planktonic growth. This was based on the laws of flow dynamics, implying that when the dilution rates within a flow cell system is higher than the μ_{max} of the fastest growing microorganism within that system, a washout of cells will occur. The dilution rate (21.15 h^{-1}) was 34 times higher than the highest specific growth rate (0.57 h^{-1}, CT03) from the bacterial isolates inoculated into the flow channels.

Nutrient conditions (type and concentration) had a definite influence on the total number and ratio of bacterial isolates found in flow channel effluent. Even though it has not been conclusively demonstrated that the planktonic cell ratios and population numbers reflect

biofilm population composition, a change in planktonic population composition can be regarded as an indication that biofilm population dynamics were influenced. Biocide treatment caused a shift either in population dominance or the disappearance of a bacterial population. The combination of nutrient condition and biocide treatment showed that the nutrient condition not only determines the antimicrobial susceptibility of a biofilm population, but also the rate of regrowth. Interestingly, the rate of regrowth of planktonic cell numbers did not reflect overall biofilm recovery. For example, it was observed that the biofilms appeared viable and active after biocide treatment, while corresponding planktonic cell numbers were low. In other instances, the biofilms appeared mostly non-viable after biocide treatment, while the viability in the corresponding planktonic community had recovered to original values. Overall, it was observed that the recovery rate of planktonic communities was much faster than the corresponding biofilms.

A model was proposed to explain this unexpected observation on biofilm-planktonic community interactions. It has been observed by Sternberg et al (1999) and Sauer et al (2000) that the highest biofilm cell activity occurred at the biofilm surfaces exposed to nutrients and not in the deeper biofilm levels or microcolonies. From these observations and the results obtained in the present study, it was proposed that at the biofilm surface, a high cell turnover takes place, which is the primary source of the associated planktonic community. When exposed to an antimicrobial substance, most of these cells at the outer region of the biofilm are killed, thereby minimizing cell turnover. However, this is the region where cell recovery is initiated when conditions become favorable again, contributing to the rapid recovery observed for the planktonic community. In deeper zones of the biofilm, dead and damaged cells need to be degraded or removed before overall biofilm recovery can be observed. The results of this study demonstrated that the rate of biofilm recovery is strongly influenced by nutrient conditions. Furthermore, the results suggest that activity in the outer layer of the biofilm, and not necessarily the overall biofilm activity, determines the number of planktonic cells in continues flow systems.

Considering the applied (e.g. biofouling control) and fundamental (comparison of planktonic and sessile phenotypes) nature of the observations made in this study on planktonic – sessile interrelationships, additional research in this area will be of value.

REFERENCES

Allison, D.G., McBrain, A.J., and Gilbert, P. (2000). Biofilms: problems of control. In: Community Structure and Co-operation in Biofilms. Lappin-Scott, H.M., Gilbert, P., Wilsion, M., and Allison, D. (eds.). SGM symposium 59. Cambridge University Press, pp. 309-327.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang J., Zhang Z., Miller W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389-3402.

Amann, R.I., Krumholz, L., and Stahl, D.A. (1990a). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172: 762-770.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., and Stahl, D. A. (1990b). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56: 1919-1925.

Amann, R. I., Ludwig, W., and Schleifer, K.-H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Reviews. 59: 143-169.

Amann, R., and Kuhl, M. (1998). In *situ* methods for assessment of microorganisms and their activities. Curr. Opin. Microbiol. 1: 352-358.

Amann, R., Fuchs, B.M., and Behrens, S. (2001). The identification of microorganisms by fluorescence in situ hybridisation. Curr. Opin. Biotechnol. 12: 231-136.

Anderl, J.N., Zahler, J., Roe, F., and Stewart, P.S. (2003). Role of nutrient limitation and stationary-phase existence in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial. Agents. Chemother. 47: 1251-1256.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1993). Current Protocols in molecular biology. Wiley Interscience, New York.

Avaniss-Aghajani, E., Jones, K., Holtzman, A., Aronson, T., Glover, N., Boian, M., Froman, S., and Brunk, C.F. (1996). Molecular techniques of rapid identification of mycobacteria. J. Clin. Microbiol. 34: 98-102.

Azeredo, L., Pacheco, A.P., Lopes, I., Oliveira, R., and Vieira, M.J. (2003). Monitoring cell detachment by surfactants in a parallel plate flow chamber. Water Sci. Technol. 47: 77-82.

Banks, M.K., and Bryers, J.D. (1991). Bacterial species dominance within a binary culture biofilm. Appl. Environ. Microbiol. 57: 1974-1979.

Behrens, S., Ruhland, C., Inacio, J., Huber, H., Fonseca, A., Spencer-Martins, I., Fuchs, B.M., and Amann R. (2003). *In situ* accessibility of small-subunit rRNA of members of the domains Bacteria, Archaea, and Eucarya to Cy3-labeled oligonucleotide probes. Appl. Environ. Microbiol. 69: 1748-1758.

Birmingham, J.J., Hughes, N.P., and Treloar, R. (1995). Diffusion and binding measurements within oral biofilms using fluorescence photobleaching recovery methods. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences. 350 (1334): 325-343.

Blackwood, C.B., Marsh, T., Kim, S-H., and Paul, E.A. (2003). Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Appl. Environ. Microbiol. 69: 926-932.

Bonet, R., Simon-Pujol, M.D., and Congregado, F. (1993). Effects of nutrients on exopolysaccharide production and surface properties of *Aeromonas salmonicida*. Appl. Environ. Microbiol. 59: 2437-2441.

Böckelmann, U., Manz, W., Neu, T.R., and Szewzyk, U. (2000). Characterization of the microbial community of lotic organic aggregates ('river snow') in the Elbe river of Germany by cultivation and molecular methods. FEMS Microbiol. Ecol. 33: 157-170.

Boulos, L., Prevost, M., Barbeau, B., Coallier, J., Desjardins, R.J. (1999). LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. Microbiol. Methods. 37: 77-86.

Brown, M.R., Allison, D.G., and Gilbert, P. (1988). Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? J. Antimicrobial. Chemother. 22: 777-780.

Brözel, V.S., Strydom, G.M., and Cloete, T.E.E. (1995). A method for the study of *de novo* protein synthesis in *Pseudomonas aeruginosa*: after attachment. Biofouling. 8: 195-210.

Brunk, C.F., Avaniss-Aghajani, E., and Brunk, C.A. (1996). A computer analysis of primer and probe hybridization potential with bacterial small-subunit rRNA sequences. Appl. Environ. Microbiol. 62: 872-879.

Chandy, J.P., and Angles, M.L. (2001). Determination of nutrients limiting biofilm formation and the subsequent impact on disinfectant decay. Water Res. 35: 2677-2682.

Christensen, B.E., and Characklis, W.G. (1990). Physical and chemical properties of biofilms. In: Characklis, W.C. Marshall, K.C. (eds). Biofilms. Wiley, New York,. pp 93-130.

Clement, B.G., Kehl, L.E., deBord, K.L., and Kitts, C.L. (1998). Terminal restriction fragment patterns (TRFPs), a rapid, PCR-based method for the comparison of complex bacterial communities. J. Microbiol. Methods. 31: 135-142.

Cloete, T.E., Jacobs, L., and Brozel, V.S. (1998). The chemical control of biofouling in industrial water systems. Biodegradation. 9: 23-37.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., and Marrie, T. J. (1987). Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41: 435-464.

Davies, D.G., Parsek, M.R., Pearson, J.P., Iglewski, B.H., Costerton, J.W., and Greenberg, E.P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science. 280: 295-298.

DeLong, E.F., Wickham, G.S., and Pace, N.R. (1989). Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science. 243: 1360-1363.

Desai, M., Bühler, T., Weller, P.H., and Brown, M.R.W. (1998). Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazindime during exponential growth. J. Antimicrobial Chemother. 42: 153-160.

Dewanti, R., and Wong, A.C.L. (1995). Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. Int. J. Food Microbiol. 26: 147-164.

Dukan, S., and Nyström, T. (1998). Bacterial senescence: stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. Genes Dev. 12: 3431-3441.

Dunbar, J., Ticknor, L.O., and Kuske, C.R. (2000). Assessment of microbial diversity in four southwestern United States soils by 16S rRNA gene terminal restriction fragment analysis. Appl. Environ. Microbiol. 66: 2943-2950.

Dunbar, J., Ticknor, L.O., and Kuske, C.R. (2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl. Environ. Microbiol. 67: 190-197.

Egert, M., and Friedrich, M.W. (2003). Formation of pseudo-terminal restriction fragments, a PCR-related bias affecting terminal restriction fragment length polymorphism analysis of microbial community structure. Appl. Environ. Microbiol. 69: 2553-2562.

Eginton, P.J., Holah, J., Allison, D.G., Handley, P.S., and Gilbert, P. (1998). Changes in the strength of attachment of micro-organisms to surfaces following treatment with disinfectants and cleansing agents. Lett. Appl. Microbiol. .27: 101-105.

Evans, D.J., Allison, M.R., Brown, M.R., and Gilbert, P. (1991). Susceptibility of *Pseudomonas aeruginosa* and *Escherichia coli* biofilms towards ciprofloxacin: effect of specific growth rate. J. Antimicrobial. Chemother. 27: 177-184.

Exner, M., Tuschewitzki, G.J., and Scharnagel, J. (1987). Influence of biofilms by chemical disinfectants and mechanical cleaning. Zentralbl. Bakteriol. Mikrobiol. Hyg. [B]. 183 (5-6): 549-63.

Flemming, H-C., Wingender, J., Mayer, C., Körstegens, V. and Borchard, W. (2000). Cohesiveness in biofilm matrix polymers. In: Community Structure and Co-operation in Biofilms. Lappin-Scott, H.M., Gilbert, P., Wilsion, M., and Allison, D. (eds.), SGM symposium 59. Cambridge University Press, pp. 87-105.

Flemming, H.C. (2002). Biofouling in water systems--cases, causes and countermeasures. Appl. Microbiol. Biotechnol. 59: 629-640.

Foley, I., Marsh, P., Wellington, E.M.H., Smith, A.W., and Brown, M.R.W. (1999). General stress response master regulator *rpo*S is expressed in human infection: a possible role in chronicity. J. Antimicrobial. Chemother. 43: 164-165.

Fuchs, B.M., Wallner, G., Beisker, W., Schwippl, I., Ludwig, W., and Amann, R. (1998). Flow cytometric analysis of the *in situ* accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl. Environ. Microbiol. 64: 4973-4982.

Fuchs, B.M., Zubkov, M.V., Sahm, K., Burkill, P.H., and Amann, R. (2000). Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. Environ. Microbiol. 2: 191-201.

Garland, J.L., Cook, K.L., Adams, J.L., and Kerkhof, L. (2001). Culturability as an indicator of succession in microbial communities. Microbial. Ecol. 42: 150-158.

Genevaux, P., Bauda, P., DuBow, M.S., and Oudega, B. (1999). Identification of Tn10 insertions in the *rfaG, rfaP,* and *galU* genes involved in lipopolysaccharide core biosynthesis that affect *Escherichia coli* adhesion. Arch. Microbiol. 172: 1-8.

Gerdes, K. (2000). Toxin-antitoxin modules may regulate synthesis of macromolecules during nutritional stress. J. Bacteriol. 182: 561-72.

Gilbert, P., Collier, P.J., and Brown, M.R. (1990). Influence of growth rate on susceptibility to antimicrobial agents: Biofilms, cell cycle, dormancy, and stringent response. Antimicrob. Agents Chemother. 34: 1865-1868.

Giovannoni, S.J., DeLong, E.F., Olsen, G.J., and Pace, N.R. (1988). Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. 170: 720-726.

Giovannoni, S.J., Britschgi, T.B., Moyer C.L., and Field, K.G. (1990). Genetic diversity in Sargasso sea bacteriaplankton. Nature. 345: 60-63.

Glöckner, F.O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebeslius, K., and Schleifer, K-H. (1996). An *in situ* hybridization protocol for detection and identification of planktonic bacteria. System. Appl. Microbiol. 19: 403-406.

Gunasekera, T.S., Sorensen, A., Attfield, P.V., Sorensen, S.J., and Veal, DA. (2002). Inducible gene expression by nonculturable bacteria in milk after pasteurization. Appl. Environ. Microbiol. 68: 1988-1993.

Hall, T.A. (1999). DNAMAN (Version 4.1) package from Lynnon Biosoft, and BioEdit Sequence Alignment Editior Coypright®. Nucl. Acids. Symp. Ser. 41: 95-98.

Heim, S., Del Mar Lleo, M., Bonato, B., Guzman, C.A., and Canepari, P. (2002). The viable but nonculturable state and starvation are different stress response of *Enterococcus faecalis*, as determined by proteome analysis. J. Bacteriol. 184:6739-6745.

Huang, C-T., Xu, K.D., McFeters, G.A., and Stewart, P.S. (1998). Spatial patterns of alkaline phosphatase expression within bacterial colony and biofilms in response to phosphate starvation. Appl. Environ. Microbiol. 64: 1526-1531.

James, G.A., Korber, D.R., Caldwell, D.E., and Costerton, J.W. (1995). Digital image analysis of growth and starvation responses of a surface-colonizing *Acinetobacter* sp. J. Bacteriol. 177: 907-915.

Karthikeyan, S., Korber, D.R., Wolfaardt, G.M., and Caldwell, D.E. (1999). Functional and structural responses of a degradative microbial community to substrates with varying degrees of complexity in chemical structure. Microb. Ecol. 38: 215-224.

Karthikeyan, S., Korber, D.R., Wolfaardt, G.M., and Caldwell, D.E. (2001). Adaptation of bacterial communities to environmental transitions from labile to refractory substrates. Int. Microbiol. 4: 73-80.

Korber, D. R., A. Choi, G. M. Wolfaardt, S. C. Ingham and D. E. Caldwell (1997). Substratum topography influences susceptibility of *Salmonella enteritidis* biofilms to trisodium phosphate. Appl. Environ. Microbiol. 63: 3352-3358.

Kreft, J-U., and Wimpenny, J.W.T. (2001). Effect of EPS on biofilm structure and function as revealed by an individual-based model of biofilm growth. Wat. Sci. Technol. 43: 135-141.

Lane, D.J., Stahl, D.A., Ohlsen, G.J., Heller, D.J., and Pace, N.R. (1985). Phylogenetic analysis of the genera *Thiobacillus* and *Thiomicrospira* by 5S rRNA sequences. J. Bacteriol. 163: 75-81.

Lawrence, J.R., Korber, K.R., and Wolfaardt, G.M. (1996). Heterogeneity of natural biofilm communities. Cells and Materials. 6: 175-191.

LeChevallier, M.W., Hassenauer, .T.S., Camper, A.K., and McFeters, G.A. (1984). Disinfection of bacteria attached to granular activated carbon. Appl. Environ. Microbiol. 48: 918-923.

LeChevallier, M.W., Cawthon, C.D., and Lee, R.G. (1988). Inactivation of biofilm bacteria. Appl. Environ. Microbiol. 54: 2492-2499.

Lee, D-H., Zo, Y.G., and Kim, S-J. (1996). Nonradioactive method to study genetic profiles of natural bacterial communities by PCR-single-strand-conformation polymorphism. Appl. Environ. Microbiol. 62: 3112-3120.

Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K-H., and Wagner, M. (1999). Combination of fluorescent *in situ* hybridization and microautoradiography-a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. 65: 1289-1297.

Lewis, K. (2000). Programmed death in bacteria. Microbiol. Mol. Biol. Rev. 64: 503-514.

Lewis, K. (2001). Minireview: The riddle of biofilm resistance. Antimicrob. Agents. Chemother. 459: 999-1007.

Liu, W-T., Marsh, T. L., Cheng, H., and Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphism of genes encoding 16S rRNA. Appl. Environ. Microbiol. 63: 4516-4522.

Looijesteijn, P.J., Boels, I.C., Kleerebezem, M., and Hugenholtz, J. (1999). Regulation of exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* by the sugar source. Appl. Environ. Microbiol. 65: 5003-5008.

Lowder, M., Unge, A., Maraha, N., Jansson, J.K., Swiggett, J., and Oliver, J.D. (2000). Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged *Pseudomonas fluorescens* A506. Appl. Environ. Microbiol. 66: 3160-3165.

Lueders, T., and Friedrich, M. W. (2003). Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and *mcrA* genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. Appl. Environ. Microbiol. 69: 320-326.

MacDonald, R., Santa, M., and Brozel, V.S. (2000). The response of a bacterial biofilm community in a simulated industrial cooling water system to treatment with an anionic dispersant. J. Appl. Microbiol. 89: 225-235.

Mader, S.S. (1996). Biology. CJ Mills(ed). Wm. C. Brown Publishers. pp 860-861.

Mah, T-F.C., and O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9: 34-39.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T. Jr., Saxman, P.R., Stredwick, J.M., Garrity, G.M., Li, B., Olsen, G.J., Pramanik, S., Schmidt, T.M., and Tiedje, J.M. (2000). The RDP (Ribosomal Database Project) continues. Nucleic Acids Res. 28: 173-174.

Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T. Jr., Saxman, P.R., Stredwick, J.M., Garrity, G.M., Li, B., Olsen, G.J., Pramanik, S., Schmidt, T.M., and Tiedje, J.M. (2001). The RDP-II (Ribosomal Database Project). Nucleic Acids Res. 29:173-174.

Maira-Litran, T., Allison, D.G., and Gilbert, P. (2000). An evaluation of the potential of the multiple antibiotic resistance operon (*mar*) and the multidrug efflux pump *acrAB* to moderate resistance towards ciprofloxacin in *Escherichia coli* biofilms. J. Antimicrob. Chemother. 45: 789-795.

Marsh, T.L. (1999). Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterization diversity among homologous populations of amplification products. Curr. Opin. Microbiol. 2: 323-327.

Marsh, T.L., Saxman, P., Cole, J., and Tiedje, J. (2000). Terminal restriction fragment length polymorphism analysis program, a web-based research too for microbial community analysis. Appl. Environ. Microbiol. 66: 3616-3620.

Maruyama, A., and Sunamura, M. (2000). Simultaneous direct counting of total and specific microbial cells in seawater, using a deep-sea microbe as target. Appl. Environ. Microbiol. 66: 2211-2215.

Mathieu-Daudé, F., Welsch, J., Vogt, T., and McClelland, M. (1996). DNA rehybridization during PCR: the 'C_0t effect' and its consequences. Nucleic Acids Res. 24: 2080-2086.

McDonnell, G., and Russell, A.D. (1999). Antiseptics and disinfectants: Activity, Action, and Resistance. Clin. Microbiol. Rev. 12: 147-179.

McLoed, G.I., and Spector, M.P. (1996). Starvation and Stationary-Phase-induced resistance to the antimicrobial peptide polymyxin B in *Salmonella typhimurium* is RpoS (σ^S) independent and occurs though both *phoP*-dependent and -independent pathways. J. Bacteriol. 178: 3683-3688.

Mitchison, J.M. (1969). Enzyme synthesis in synchronous cultures. Science. 165: 657-663.

Moeseneder, M.M., Arrieta, J. M., Muyzer, G., Winter, C., and Herndl, G. J. (1999). Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Appl. Environ. Microbiol. 65: 3518-3525.

Moller, S., Korber, D.R., Wolfaardt, G.M., Molin, S., and Caldwell, D.E. (1997). Impact of nutrient composition on a degradative biofilm community. Appl. Environ. Microbiol. 63: 2432-2438.

Morton, L.H.G., Greenway, D.L.A., Gaylarde, C.C., and Surman, S.B. (1998). Consideration of some implications of the resistance of biofilms to biocides. Int. Biodeterioration and Biodegradation. 41: 247-259.

Moter, A., and Göbel, U.B. (2000). Fluorescence *in situ* hybridization (FISH) for direct visualization of microorganisms. J. Microbiol. Methods. 41: 85-112.

Moyer, C.L., Dobbs, F.C., and Karl, D.M. (1994). Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system. Loihi seamount. Hawaii. Appl. Environ. Microbiol. 60: 871-879.

Moyer, C.L., Tiedje, J.M., Dobbs, F.C., and Karl, D.M. (1996). A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. Appl. Environ. Microbiol. 62: 2501-2507.

Muyzer, G., de Waal, F.C., and Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S RNA. Appl. Environ. Microbiol. 59: 695-700.

Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. Curr. Opin. Microbiol. 2: 317-322.

Nichols, W.W. (1989). Susceptibility of biofilms to toxic compounds. Structure and function of biofilms. Characklis, W.G., and Wilderer, P.A. (eds.), John Wiley & Sons Ltd. pp 321-331.

Nickel, J.C., Ruseska, I., Wright, J.B., and Costerton, J.W. (1985) Tobramycin resistance of *Pseudomonas aeruginosa* cells growing as a biofilm on urinary catheter material. Antimicrob. Agents Chemother. 27: 619-624.

Norton, C.D., and LeChevallier, M.W. (2000). A pilot study of bacteriological population changes through potable water treatment and distribution. Appl. Environ. Microbiol. 66: 268-276.

Nyström, T. (2003). Nonculturable bacteria: programmed survival forms or cells at death's door? Bioessays. 25: 204-211.

Olsen, G.J., Lane, D.J., Giovannoni, S.J., Pace, N.R., and Stahl, D.A. (1986). Microbial ecology and evolution: a ribosomal RNA approach. Annu. Rev. Microbiol. 40: 337-365.

Pace, N.R., Stahl, D.A., Lane, D.J., and Olsen, G.J. (1986). The analysis of natural microbial poulations by ribosomal RNA sequences. Adv. Microbial. Ecol. 9: 1-55.

Pedersen, K. (1990). Biofilm development on stainless steel and PVC surfaces in drinking water. Wat. Res. 24: 239-243.

Pedersen, K., Christensen, S.K., and Gerdes, K (2002). Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. 45: 501-510.

Polz, M. F., and Cavanaugh, C. M. (1998). Bias in template-to-product rations in multitemplate PCR. Appl. Environ. Microbiol. 64: 3724-3730.

Prigent-Combaret, C., Vidal, O., Dorel, C., and Lejeune, P. (1999). Abiotic surface sensing and biofilm dependent regulation of gene expression in *Escherichia coli*. J. Bacteriol. 18: 5993-6002.

Rawlings, D.E. (1995). Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* strains in leaching environments. In: Biohydrometallurgical processing Vol II.. Jerez, C.A., Vargas, T., Toledo, H., and Wiertz, J.V. (eds). University of Chile, Chile. pp 9-17.

Rickard, A.H., Leach, S.A., Buswell, C.M., High, N.J., and Handley, P.S. (2000). Coaggregation between aquatic bacteria is mediated by specific-growth-phase-dependent lectin-saccharide interactions. Appl. Environ. Microbiol. 66:431-434.

Rickard, A.H., McBain, A.J., Ledder, R.G., Handley, and P.S., Gilbert, P. (2003). Coaggregation between freshwater bacteria within biofilm and planktonic communities. FEMS Microbiol. Lett. 220: 133-140.

Rockabrand, D., Austin, T., Kaiser, R., and Blum, P. (1999). Bacterial growth state distinguished by single-cell protein profiling: does chlorination kill coliforms in municipal effluent? Appl. Environ. Microbiol. 65: 4181-4188.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sauer, K., and Camper, A.K. (2001). Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. J. Bacteriol. 183: 6579-6589.

Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., and Davies, D. G. (2002). *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J. Bacteriol. 184:1140-1154.

Schlegel, H. (1986). General Microbiology. Cambridge: Cambridge University Press.

Schleifer, K. H., and Stackebrandt, E. (1983). Molecular systematic of prokaryotes. Annu. Rev. Microbiol. 37: 143-187.

Schlictman, D., Kavanaugh-Black, A., Shankar, S., and Chakrabarty, A.M. (1994). Energy metabolism and alginate biosynthesis in *Pseudomonas aeruginosa*: role of the tricarboxylic acid cycle. J. Bacteriol. 176: 6023-6029.

Schmidt, T. M., DeLong, E. F., and Pace, N. R. (1991). Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. 173: 4371-4378.

Spoering, A.L., and Lewis, K. (2001). Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. J. Appl. Bacteriol. 183: 6746-6751.

Stahl, D.A., Lane, D.J., Olsen, G.J., and Pace, N.R. (1985). Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. 49:1379-1384.

Staley, J.T., and Konopka, A. (1985). Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. 39: 321-346.

States, D. J. (1992). Molecular sequence accuracy: analyzing imperfect data. Trends Genet. 8: 52-55.

Sternberg, C., Christensen, B.B., Johansen, T., Toftgaard Nielsen, A., Andersen, J.B., Givskov, M., and Molin, S. (1999). Distribution of bacterial growth activity in flow-chamber biofilms. Appl. Environ. Microbiol. 65: 4108-4117.

Stewart, P.S. (1996). Theoretical aspects of antibiotic diffusion into microbial biofilms. Antimicrobial. Agents Chemother. 40: 2517-2522.

Stewart, P.S. (2003). Diffusion in biofilms. J. Bacteriol. 185: 1485-1491.

Suzuki, M. T., Rappe, S., and Giovannoni, S. J. (1998). Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Appl. Environ. Microbiol. 64: 4522-4529.

Swift, S., Throup, J.P., Williams, P., Salmond, G.P., and Steward, G.S. (1996). Quorum sensing: a population-density component in the determination of bacterial phenotype. Trends Biochem. Sci. 21: 214-219.

Szewzyk, U., Szewzyk, R., and Stenstrom, TA. (1994). Thermophilic, anaerobic bacteria isolated from a deep borehole in granite in Sweden. Proc. Natl. Acad. Sci. USA. 91: 1810-1813.

Tiedje, M. J., Asuming-Brempong, S., Nüsslein, K., Marsh, T. L., and Flynn, S. J. (1999). Opening the black box of soil microbial diversity. Appl. Soil Ecol. 13: 109-122.

Van de Peer, Y., De Rijk, P., Wuyts, J., Winkelmans, T., and De Wachter, R. (2000). The European small subunit ribosomal RNA database. Nucleic Acids Res. 28:175-176.

Von Wintzingerode, F., Göbel, U.B., and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21: 213-229.

Ward, D.M., Weller, R., and Bateson, M.M. (1990). 16S rRNA sequences reveal numerous uncultured microoganisms in a natural community. Nature. 345: 63-65.

Watnick, P., and Kolter, R. (2000). Biofilm, city of microbes. J. Bacteriol. 182: 2675-269.

Whiteley, M., Parsek, M.R., and Greenberg, E.P. (2000). Regulation of quorum sensing by *RpoS* in *Pseudomonas aeruginosa*. J. Bacteriol. 182: 4356-4360.

Whiteley, M., Ott, J. R., Weaver, E.A., and McLean, R.J.C. (2001). Effects of community composition and growth rate on aquifer biofilm bacteria and their susceptibility to betadine disinfections. Environ. Microbiol. 3: 43-52.

Wingender, J., Neu, T., and Flemming, H-C. (1999). What are bacterial extracellular polymer substances? In: Microbial Extracellular Polymeric Substances. Wingender, J., Neu, T., and Flemming, H.-C. (eds). Springer, Heidelberg, Berlin. pp. 1-19.

Woese, C.R. (1987). Bacterial evolution. Microbial. Rev. 51: 221-271.

Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D., Caldwell, S.J., and Caldwell, D.E. (1994a). Multicellular organization in a degradative biofilm community. Appl. Environ. Microbiol. 60: 434-446.

Wolfaardt, G.M., Lawrence, J.R., Robarts, R.D., Caldwell, S.J., and Caldwell, D.E. (1994b). The role of interactions, sessile growth, and nutrient amendments on the degradative efficiency of a microbial consortium. Can. J. Microbiol. 40: 331340.

Wrangstadh, M., Szewzyk, U., Ostling, J., and Kjelleberg, S. (1990). Starvation-specific formation of a peripheral exopolysaccharide by a marine *Pseudomonas* sp., strain S9. Appl. Environ. Microbiol. 56: 2065-2072.

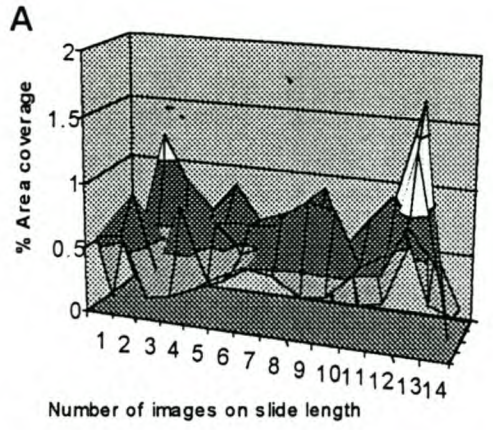
Xu, K.D., Stewart, P.S., Xia, F., Huang, C-T., and McFeters, G.A. (1998). Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. Appl. Environ. Microbiol. 64: 4035-4039.

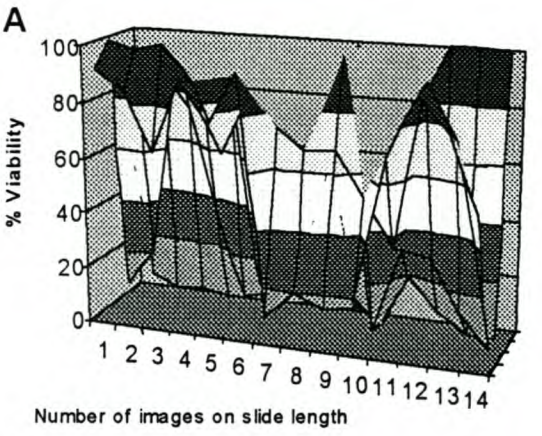
Xu, K.D., McFeters, G.A. and Stewart, P.S. (2000). Biofilm resistance to antimicrobial agents. Microbiol. 146: 547-549.

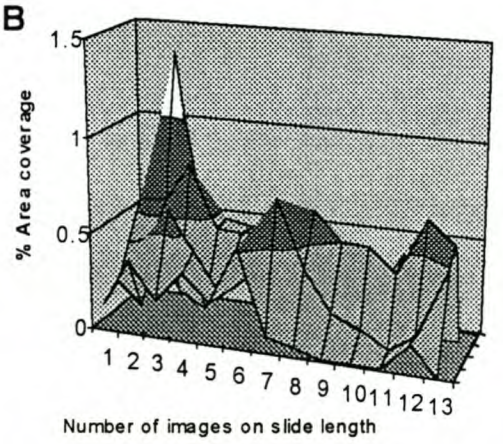
Yasuda, K., Adachi, H., Fujiwara, Y., and Ishii, N. (1999). Protein carbonyl accumulation in aging dauer formation-defective (daf) mutants of *Caenorhabditis elegans*. J. Gerontol. A. Biol. Sci. Med. Sci. B47-5; discussion B52-53.

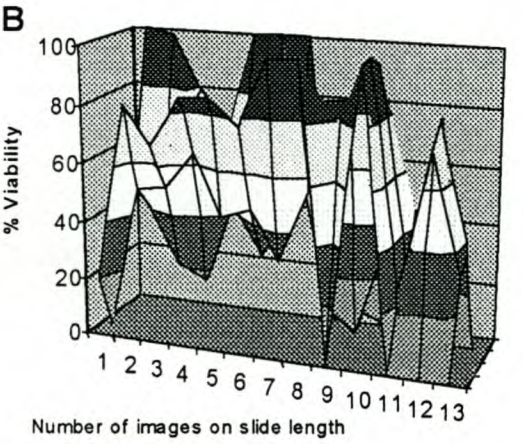
Zar, J.H. (1984). Biostatistical analysis. Prentice-Hall, London.

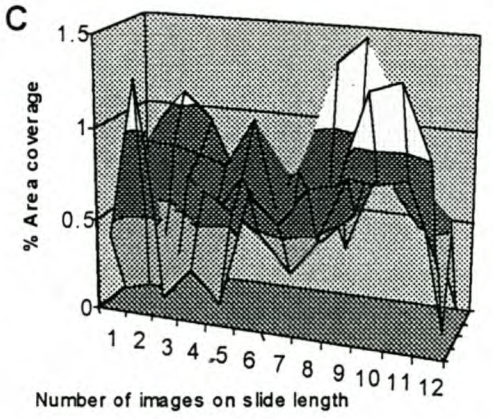
APPENDIX











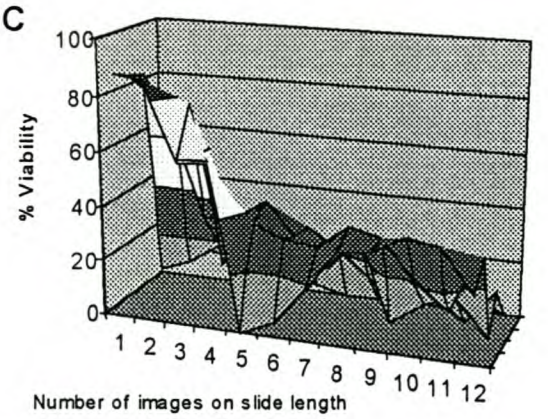


FIG 4.2.1.1. Heterogeneity in biofilm area coverage of a 4-day old biofilm was determined for two identically biocide treated (B and C)and one untreated (A) glass slide. All biofilms had a different heterogeneity pattern, even the two biocide treated biofilms.

FIG 4.2.2.1 Heterogeneity in biofilm viability of a 4-day old biofilm was determined for two identically biocide treated (B and C) and one untreated (A) glass slide. All biofilms had a different heterogeneity pattern.

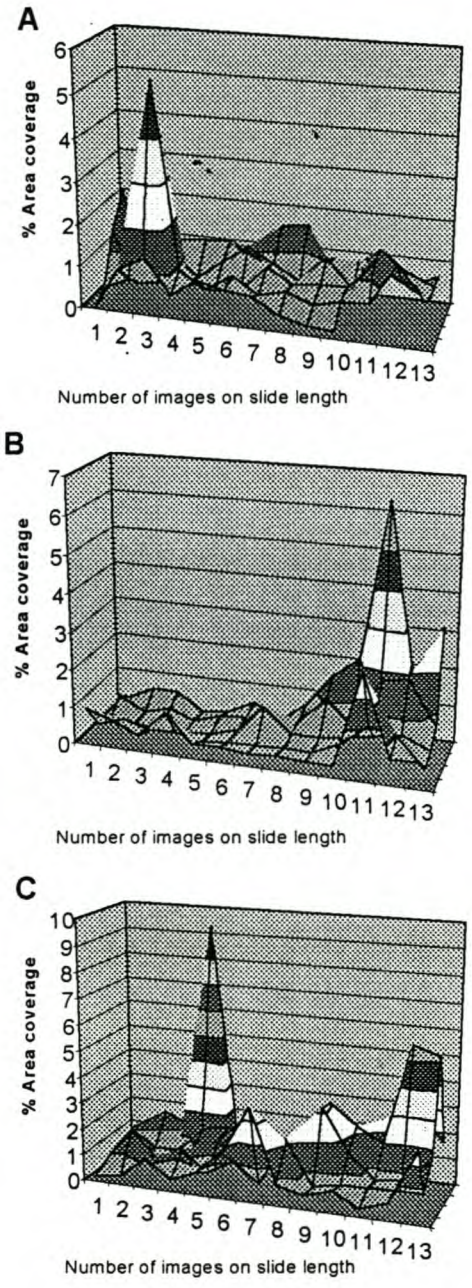


FIG 4.2.1.2. Heterogeneity in biofilm area coverage of a 7-day old biofilm was determined for two identically biocide treated (B and C) and one untreated (A) glass slide. All biofilms had a different heterogeneity pattern, even the two biocide treated biofilms.

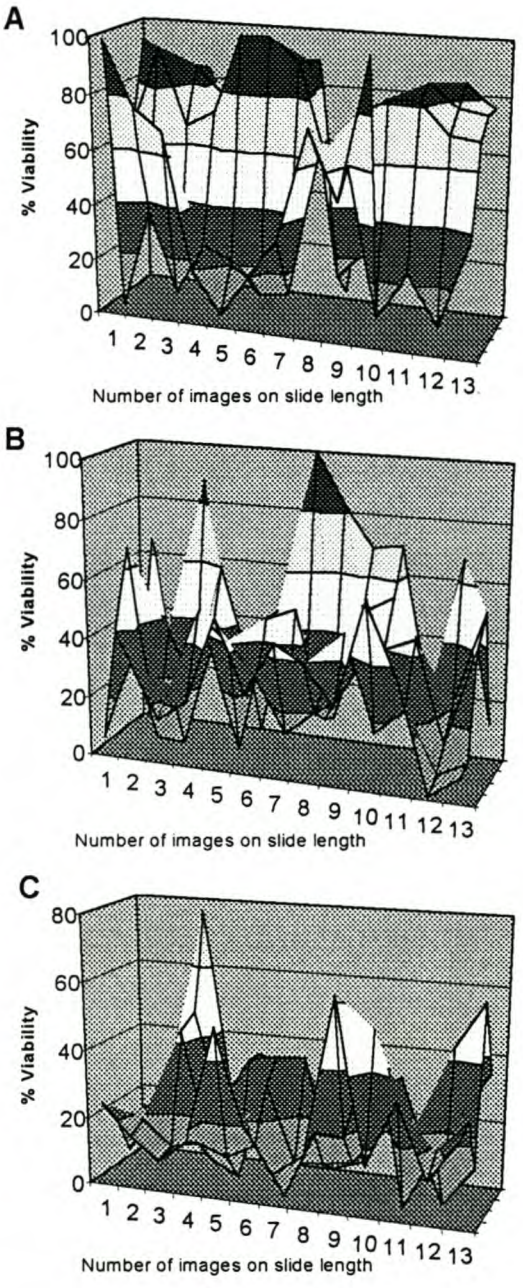
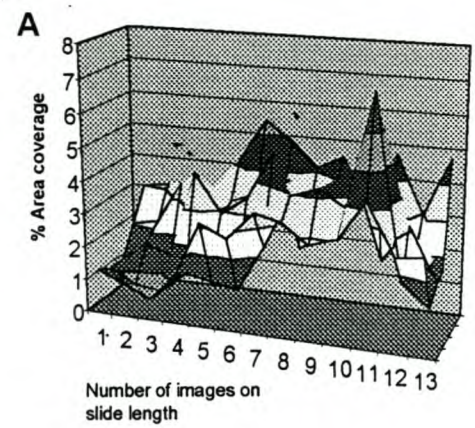
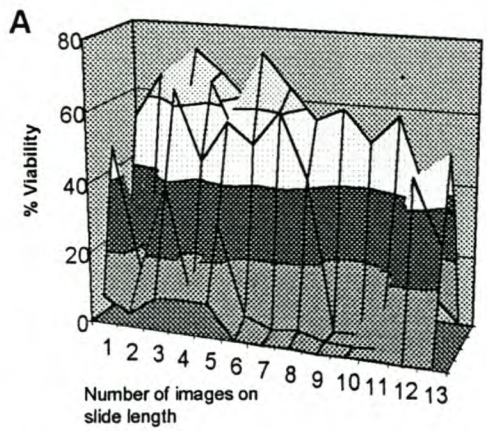
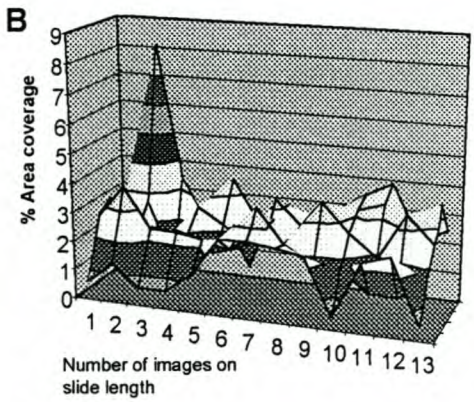
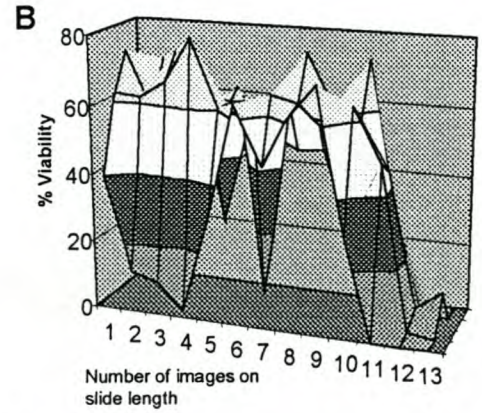


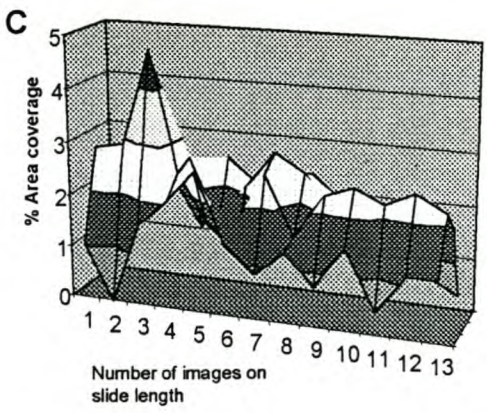
FIG 4.2.2.2. Heterogeneity in biofilm viability of a 7-day old biofilm was determined for two identically biocide treated (B and C) and one untreated (A) glass slide. All biofilms had a different heterogeneity pattern, even the two biocide treated biofilms.











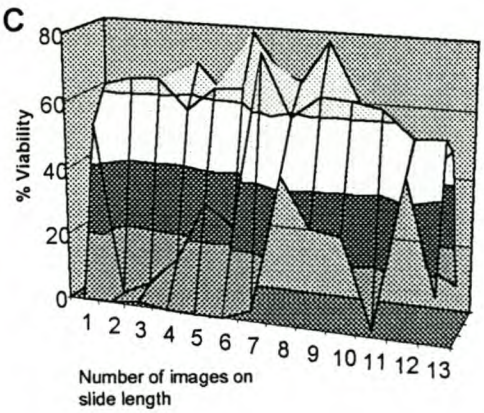


FIG 4.2.1.3 Heterogeneity in biofilm area coverage of a 14-day old was determined for two biocide treated (B and C) and one untreated (A) glass slide. All biofilms had a different heterogeneity pattern.

FIG 4.2.2.3 Heterogeneity in biofilm viability of a 14-day old was determined for two biocide treated (B and C) and one untreated (A) glass slide. All biofilms had a different heterogeneity pattern.

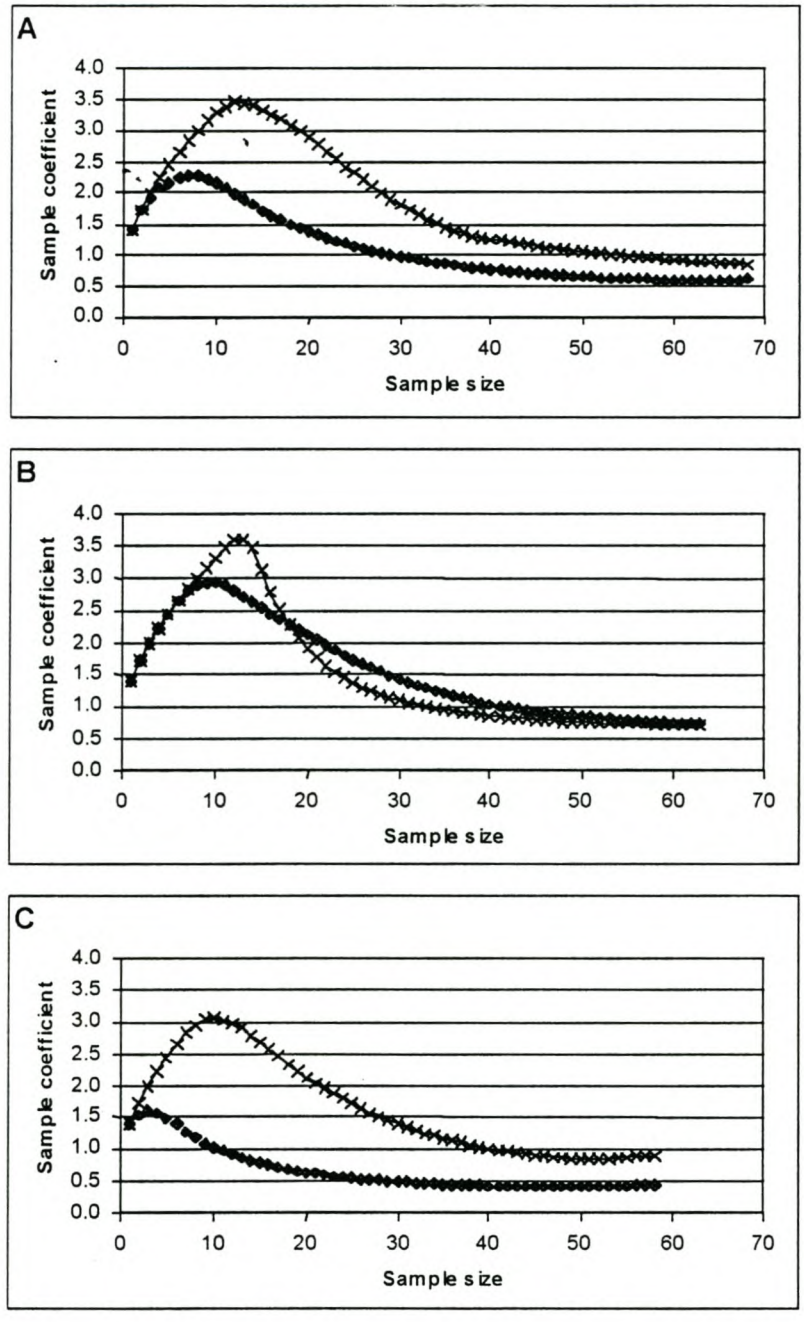


FIG 4.2. 1 Sample size of 4-day old biofilm for one untreated (A) and two identically treated (B and C) biofilm covered glass slides. Biofilm area coverage (diamond) and biofilm viability (X) sample sizes did not differ greatly between biocide-treated and untreated biofilms, with a general sample size of 40 to 60 image fields need for accurate statistical analysis.

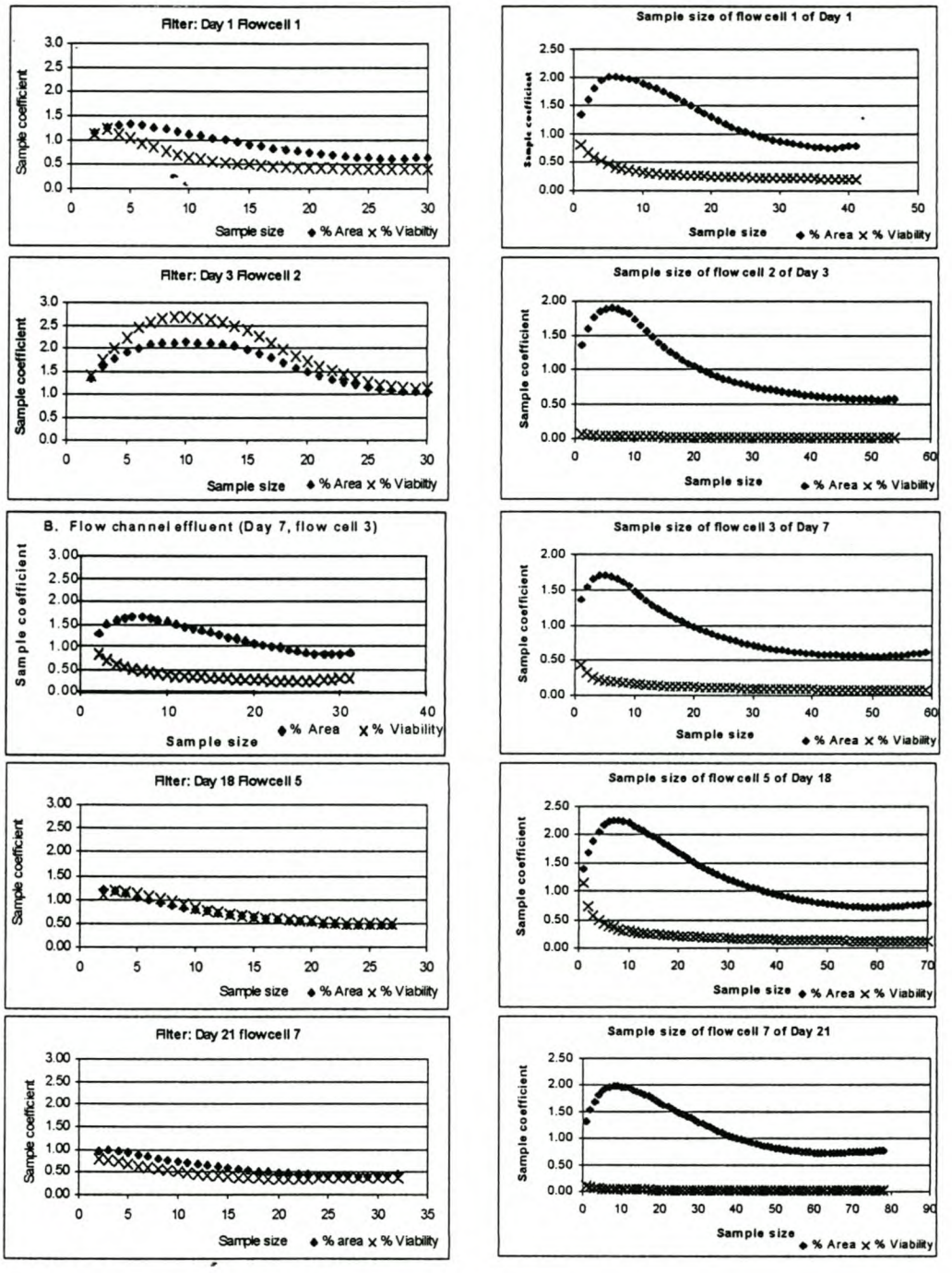


FIG 4.2.2 Sample sizes were determined for filter and biofilm (flow cell) analyses over time. The average required sample sizes for filters ranged between 20 and 30 images, while for biofilms 30 to 50 images were required.

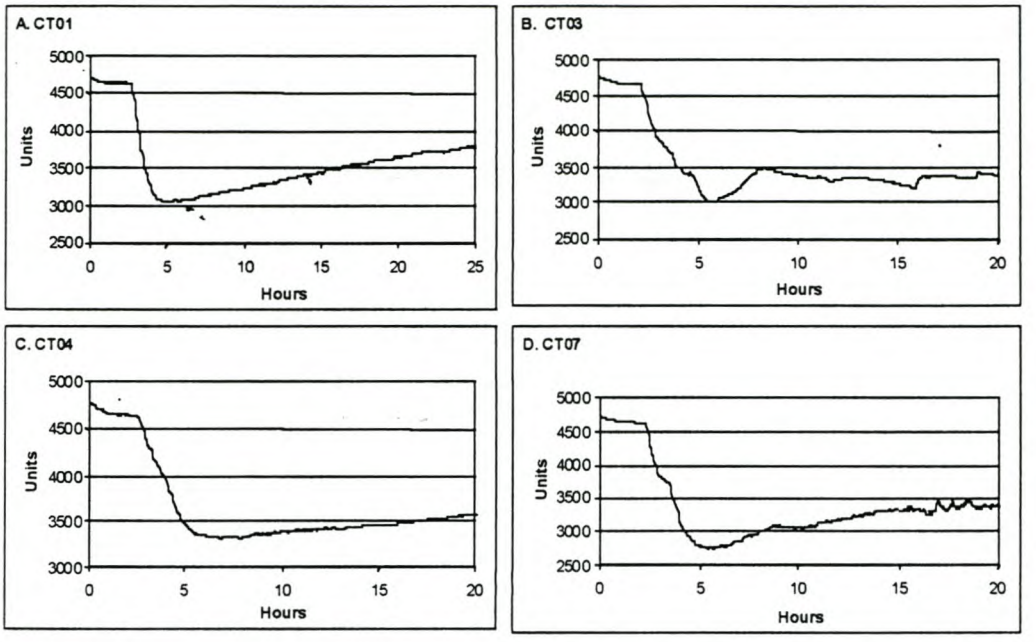


FIG 4.3.4.2 Biofilm growth curves of bacterial isolate populations, CT01 (A), CT03 (B), CT04 (C) and CT07 (D), grown under continuous flow conditions within an adapted flow cell system supplied with MSM + 0.1 g/l Glucose. Biofilm growth was monitored with the help of OLAPH (optical large area photometer).

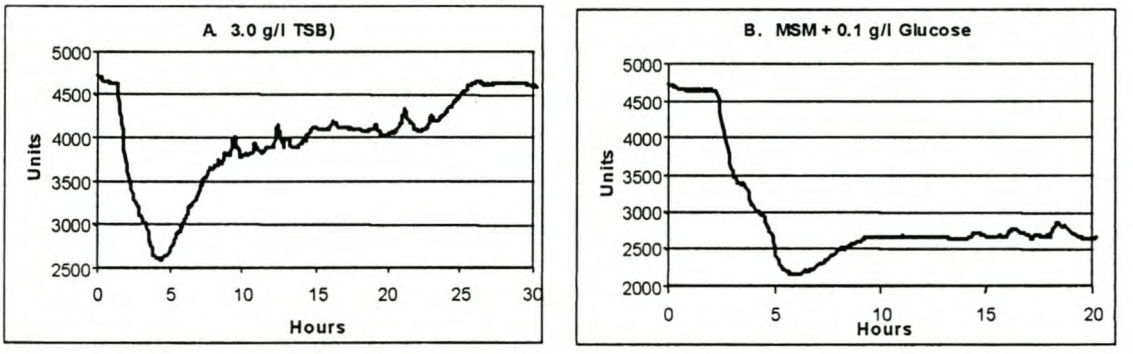


FIG 4.3.4.3 Biofilm growth curves of mixed species bacterial community, CT01 (A), CT03 (B), CT04 (C) and CT07 (D), grown under continuous flow conditions within an adapted flow cell system supplied with TSB (A) and MSM + 0.1 g/l Glucose (B). Biofilm growth was monitored with the help of OLAPH (optical large area photometer).

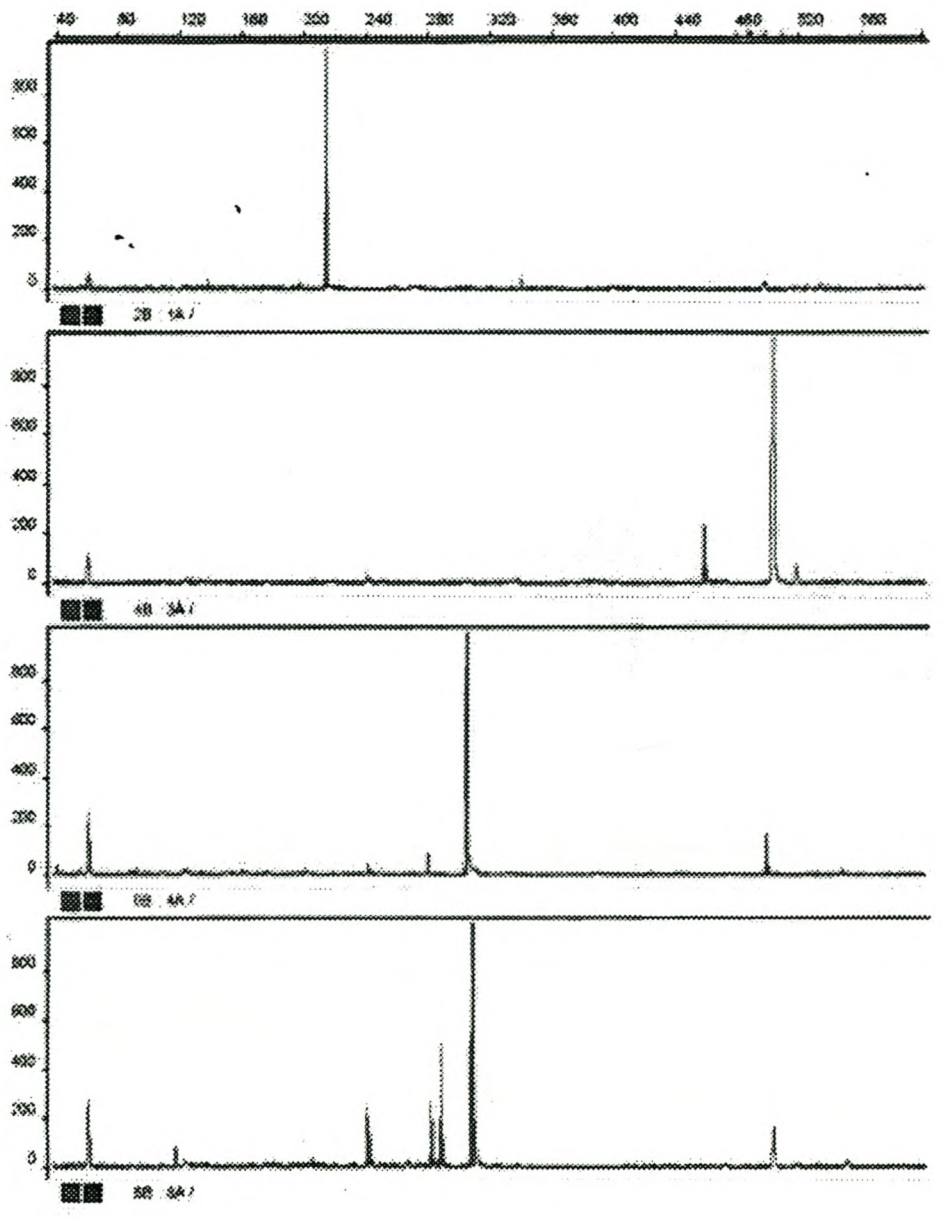


FIG 4.7.1 A T-RFLP profile of isolates CT01 (1A), CT03 (3A), CT04 (4A) and CT07 (8A) when grown overnight in 3.0 g/l TSB. Isolated 16S rDNA fragments of each isolate were cut with the restriction enzyme *Alu*I.

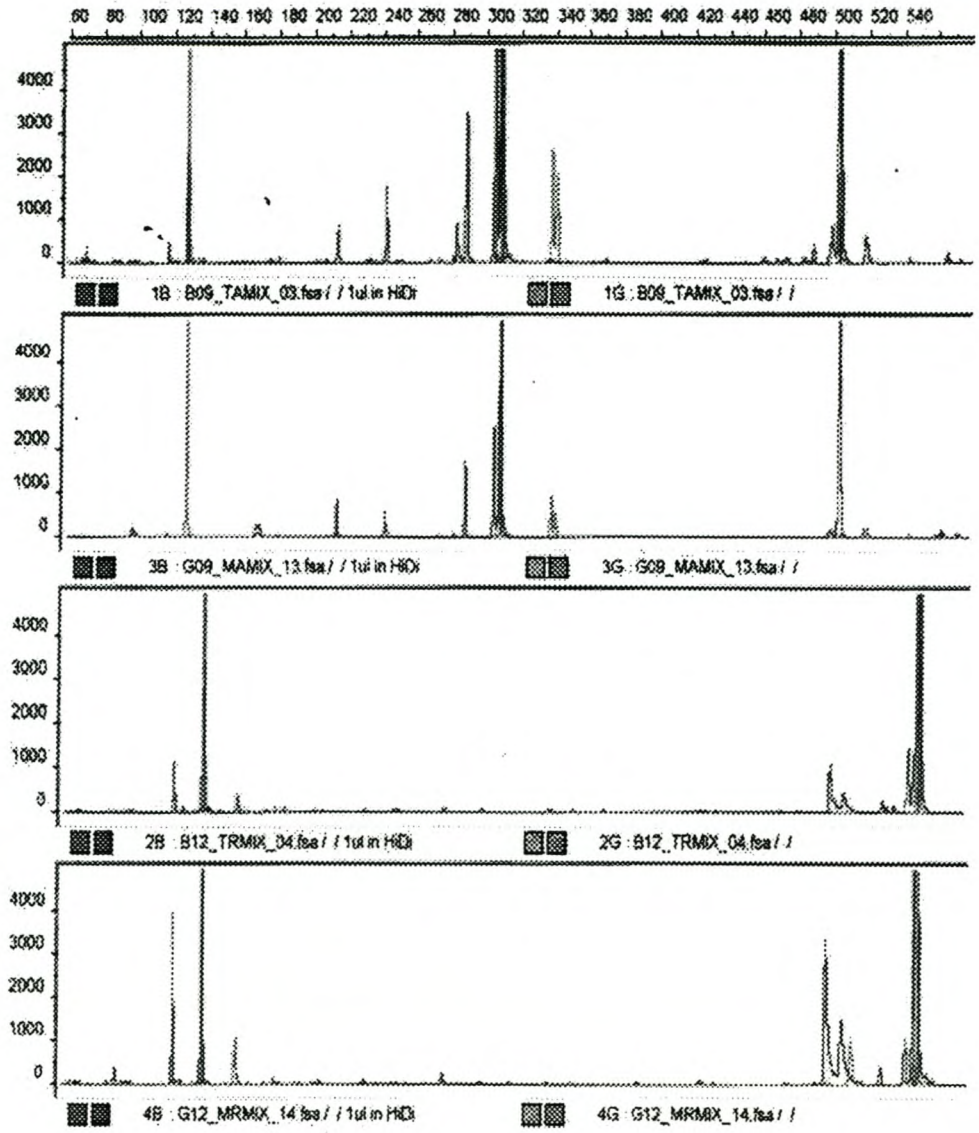


FIG 4.7.2 T-RFLP profiles of a defined mixed-species community (MIX) used to inoculate flow channels that were supplied with MSM + 0.1 g/l Glucose (M) and 3.0 g/l TSB (T). Isolated 16S rDNA fragments were cut with the restriction enzymes *Alu*I (A) and *Rsa*I (R) for terminal restriction fragment lengths. Two PCR primers were used a forward primer 314f-FAM labeled (blue) and reverse primer 1389r-HEX labeled (green).

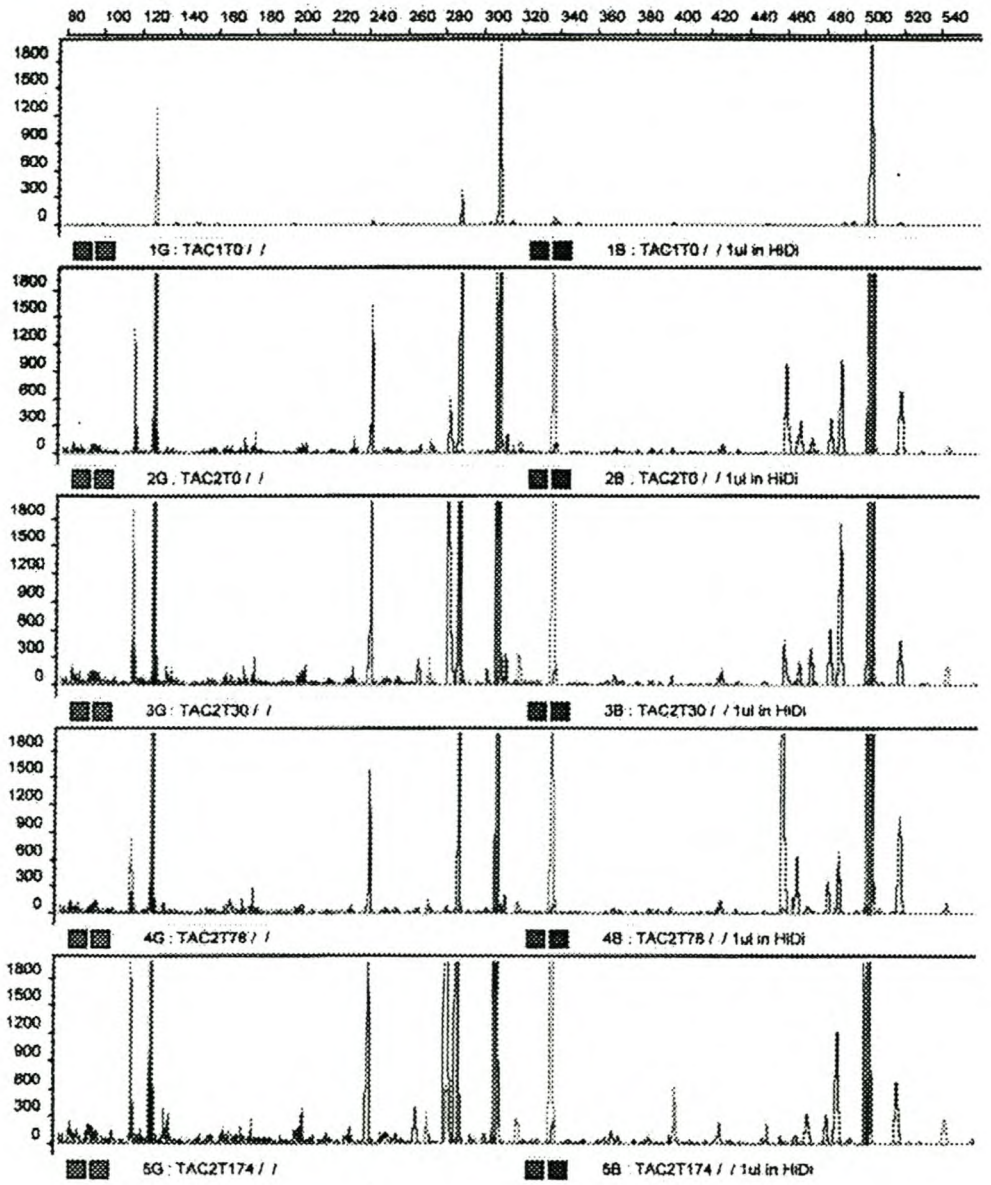


FIG 4.7.3 T-RFLP profiles of untreated planktonic communities (C2) grown with 3.0 g/l TSB (T) in a continuous flow system over time (T0 = 1 hour, T30 = 24 hours, T78 = 78 hours at 174 hours, after biocide treatment). Isolated 16S rDNA fragments were cut with the restriction enzymes *Alu*I (A) for terminal restriction fragment lengths. Two PCR primers were used a forward primer 314f-FAM labeled (blue) and reverse primer 1389r-HEX labeled (green). The community profile did not significantly change over time.

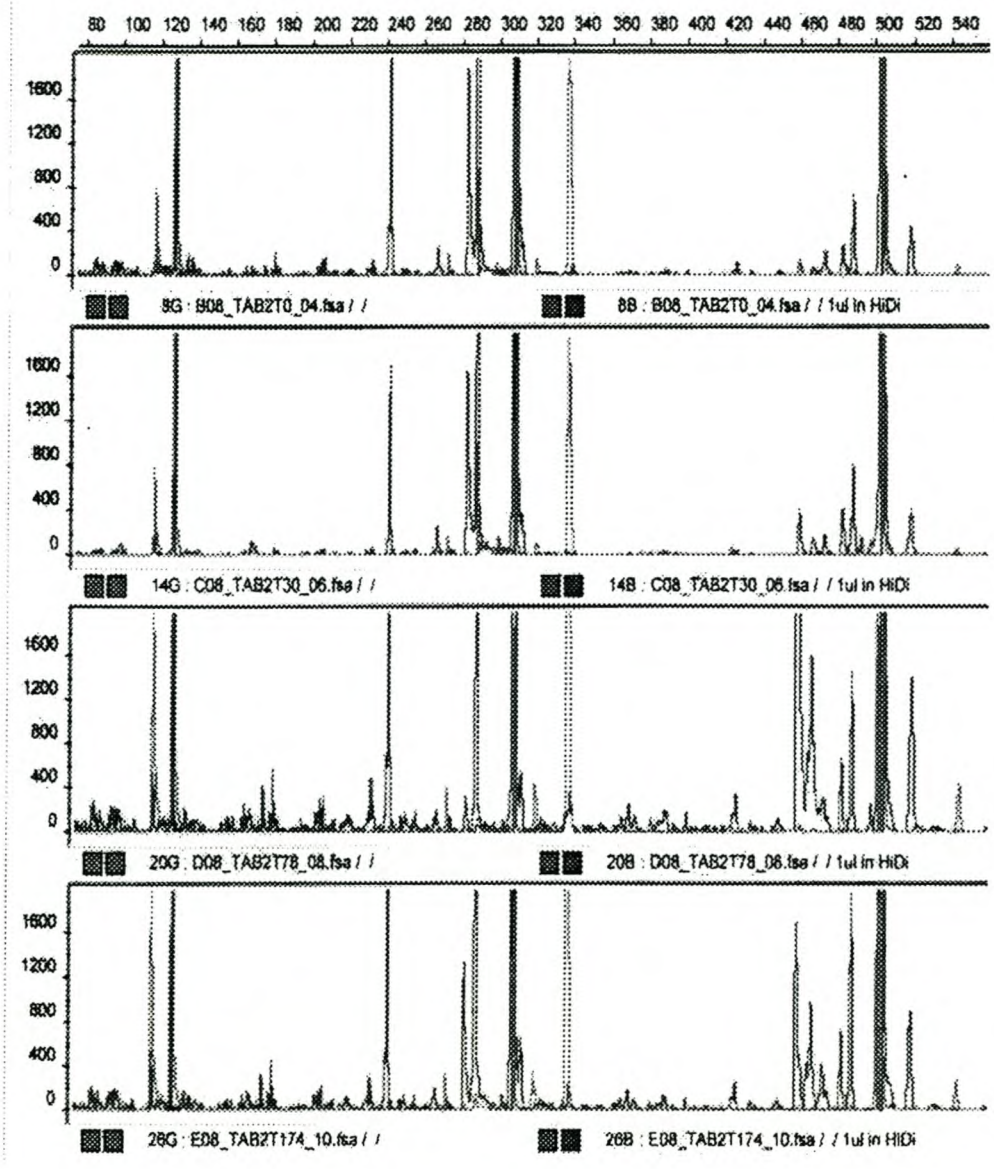


FIG 4.7.4 T-RFLP profiles of treated planktonic communities (B2) grown with 3.0 g/l TSB (T) in a continuous flow system over time (T0 = 1 hour, T30 = 24 hours, T78 = 78 hours at 174 hours, after biocide treatment). Isolated 16S rDNA fragments were cut with the restriction enzymes *Alu*I (A) for terminal restriction fragment lengths. Two PCR primers were used a forward primer 314f-FAM labeled (blue) and reverse primer 1389r-HEX labeled (green). Similar to untreated communities the profile of the treated did not significantly change over time.